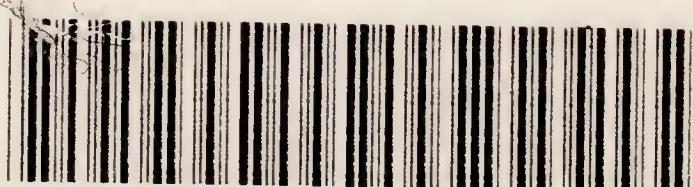


A LABORATORY MANUAL
OF
PHYSIOLOGICAL CHEMISTRY
—
E.W. ROCKWOOD, M.D., PH.D.

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A LABORATORY MANUAL
OF
PHYSIOLOGICAL CHEMISTRY

BY

ELBERT W. ROCKWOOD, M.D., Ph.D.

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FOURTH EDITION, REVISED AND ENLARGED

Illustrated With One Colored Plate, Three Plates of Microscopic Preparations and Seventeen Engravings



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PREFACE TO FOURTH EDITION.

IN a subject where rapid advancement is being made no explanation for the issue of a new edition of a textbook is necessary. Not only is the field of physiological chemistry steadily enlarging through intensive research, but the subject is being more widely studied than ever before. Although a few years ago physiological chemistry was almost entirely confined to medical curricula, it is now included not only in such courses as dentistry and pharmacy, but in those of normal, scientific and industrial colleges and of schools of home economics and domestic science.

There will be differences of opinion as to what material should be included in any text and what should be dispensed with. Because of the belief that for a first course too complete a guide is usually an embarrassment to the student, no attempt has been made to make this book all-inclusive or encyclopedic.

Most of the methods used in modern clinical chemistry are given, or at least the typical ones. Those which are chiefly employed in research alone are, for the most part, omitted, since the advanced student should go to the literature for them. More work has been outlined than can be completed in the usual medical curriculum. This has been done partly to allow the instructor some choice in methods, partly to let the exceptional student, who is found in every large class, gratify his desire to advance beyond the average limit. For some of the newer meth-

ods references have been given to the literature to enable such students to become acquainted with their origin and the facts upon which they are based. This has not been done with older and better known reactions.

Numerous questions have been inserted to challenge the student's understanding of what he has done in the laboratory. These may be answered in a note-book, or later in a review in class.

I am indebted to Mr. J. H. Crowell for a number of suggestions as to the text.

E. W. R.

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PLATE I.

1. *a*, Wheat starch granules.
b, Potato starch granules.
2. *a*, Corn starch granules.
b, Buckwheat starch granules.
3. Hemin crystals, color brown.
4. Cholesterol, colorless, transparent.
5. Phenyl glucosazone, yellow.
6. *a*, Urea, colorless.
b, Urea nitrate, colorless.

PLATE I.

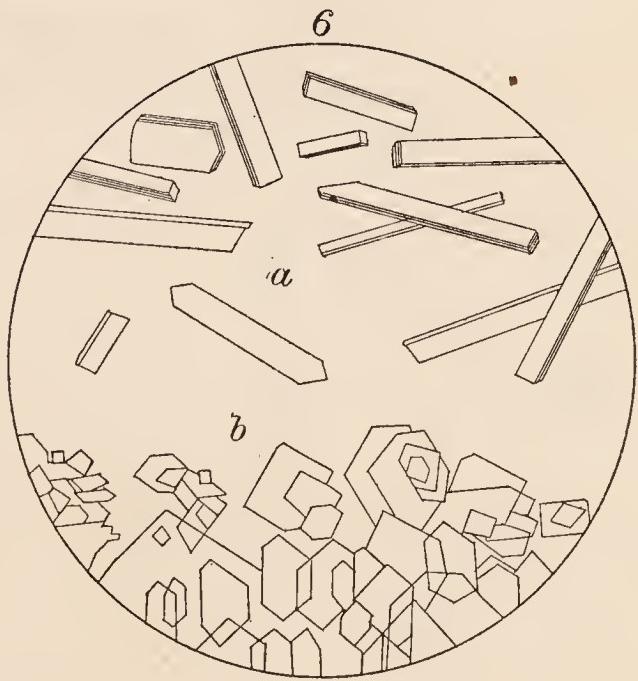
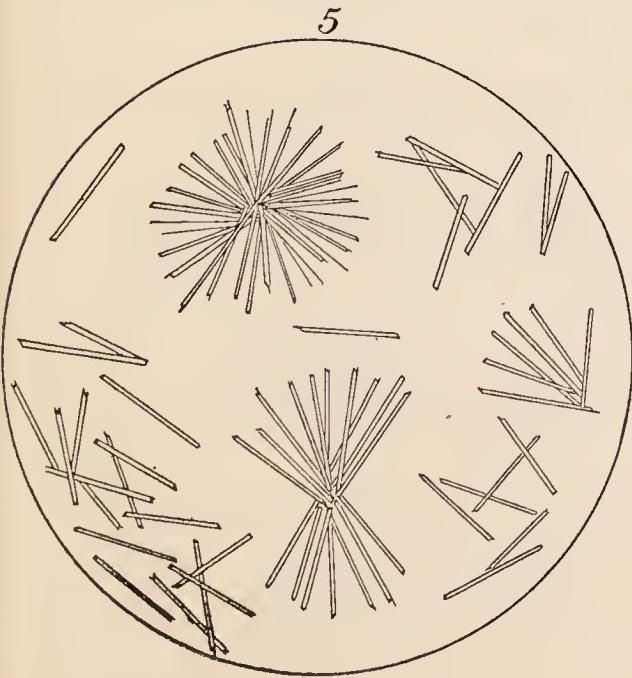
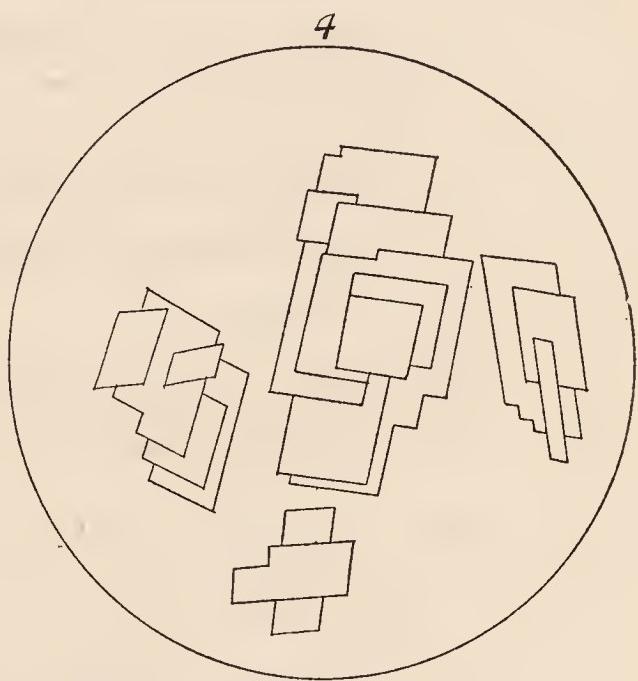
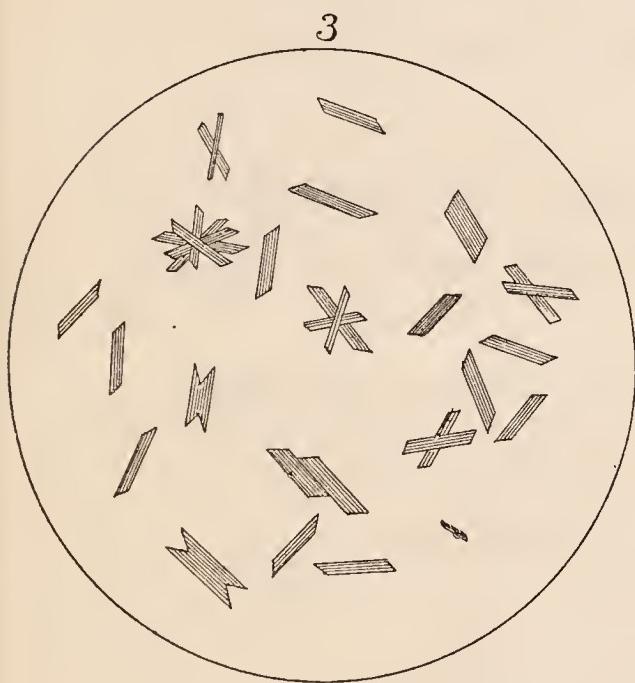
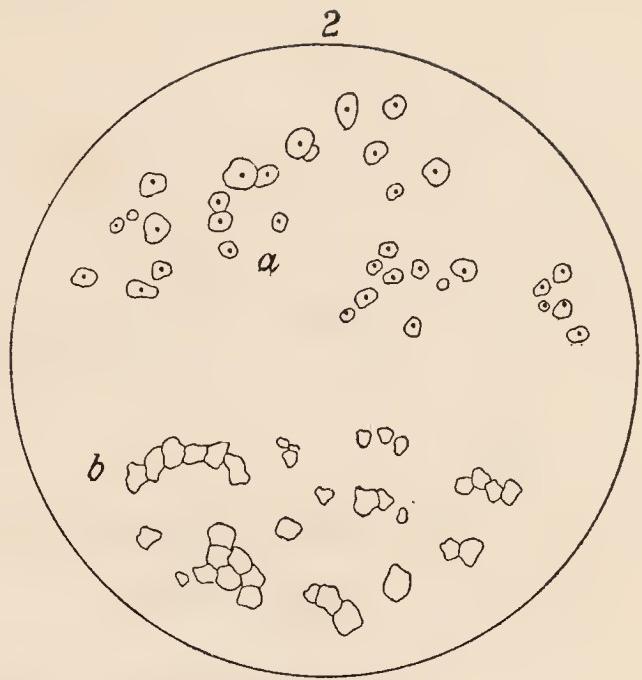
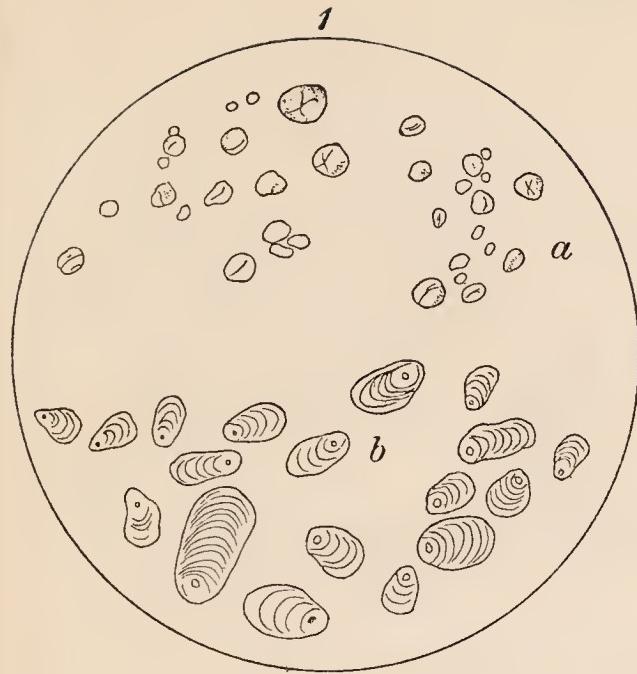


PLATE II.

7. Calcium phosphate, crystallized and amorphous forms,
both colorless.
8. Triple phosphate, "coffin lid" crystals, colorless.
Sodium urate, brown spherical masses with spicules.
Bacteria.
9. Ammonium urate, "thorn apple" forms, color brown.
Calcium carbonate, spherules and dumb bell forms,
colorless.
10. Calcium oxalate, "dumb bell" and "envelope shape"
crystals, colorless.
11. Uric acid crystals, yellow to dark brown.
Amorphous urates, brownish.
12. *a*, Calcium sulphate crystals, colorless.
b, Impure leucine, nearly colorless.
c, Tyrosine, colorless when pure.

PLATE II.

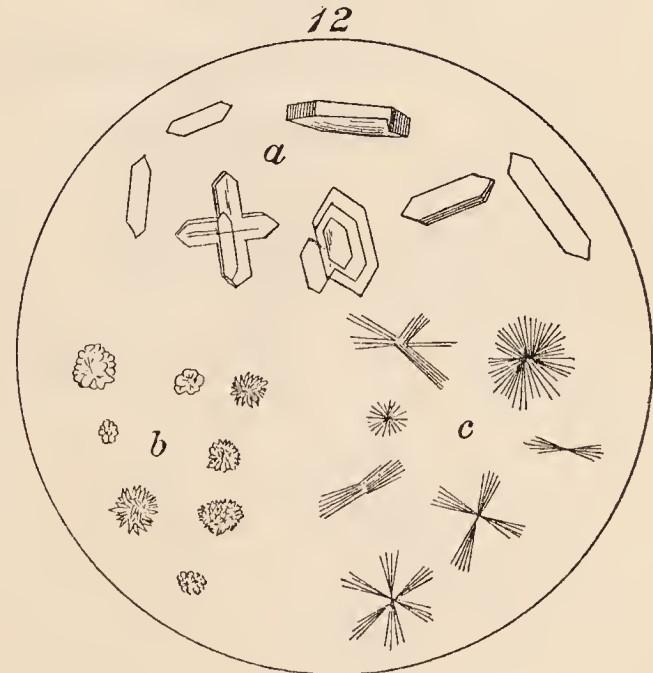
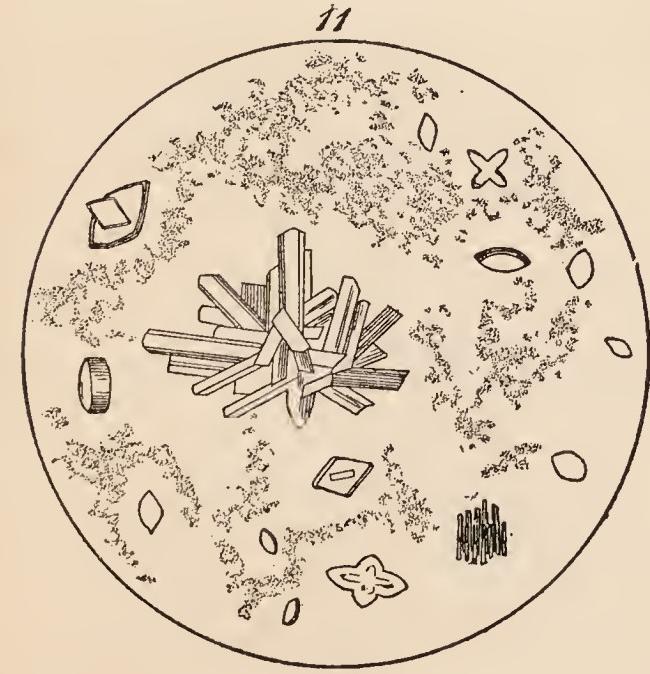
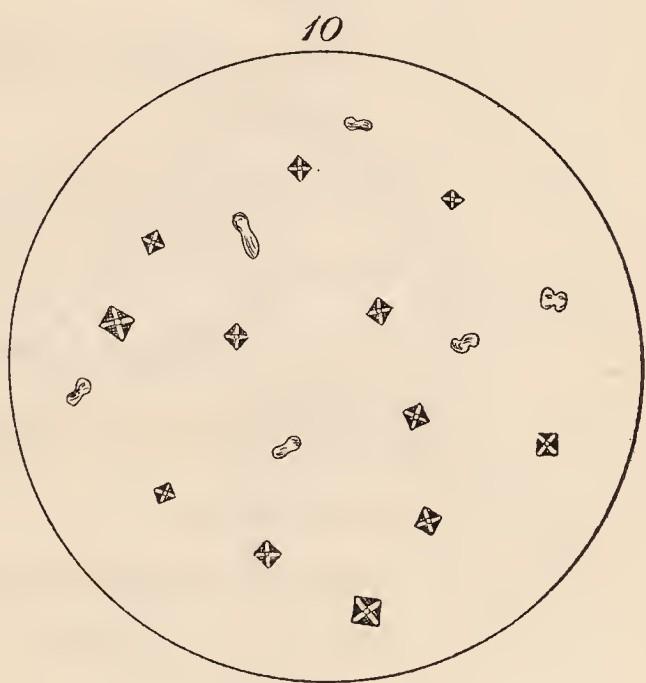
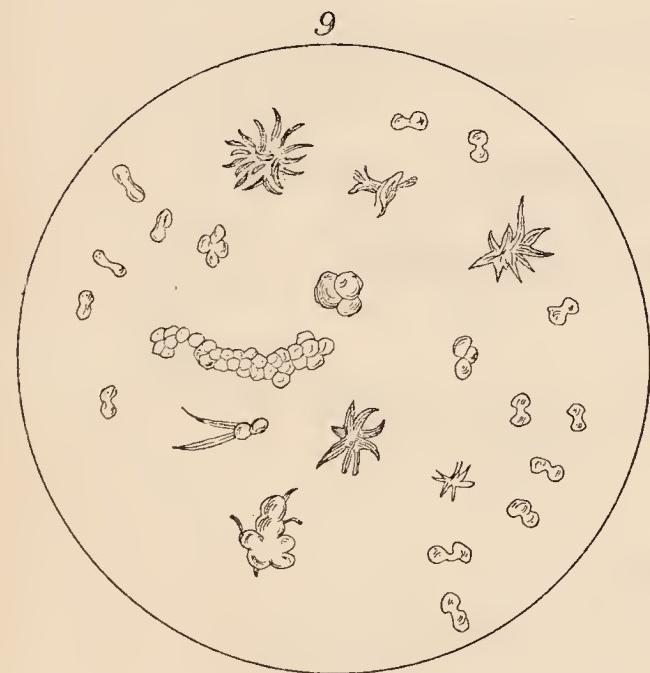
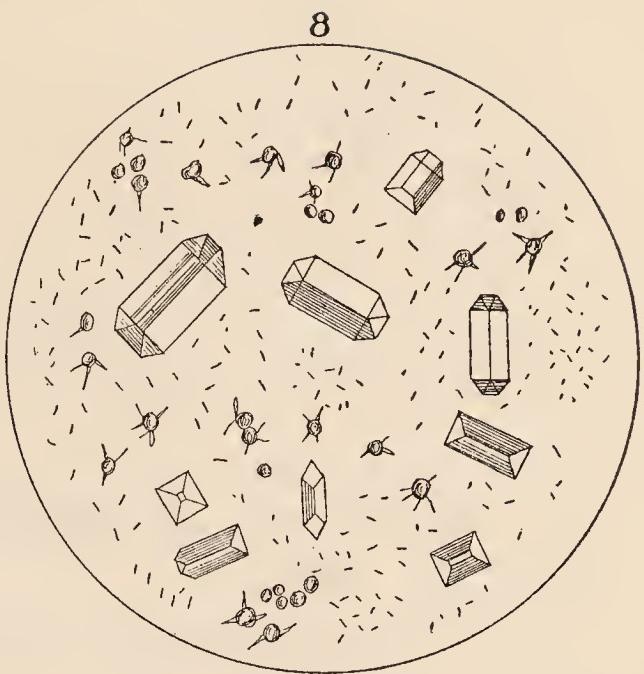
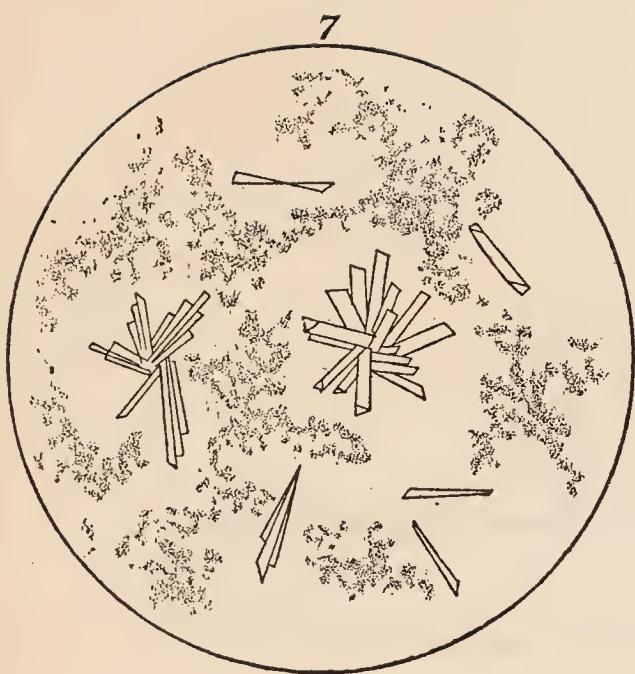
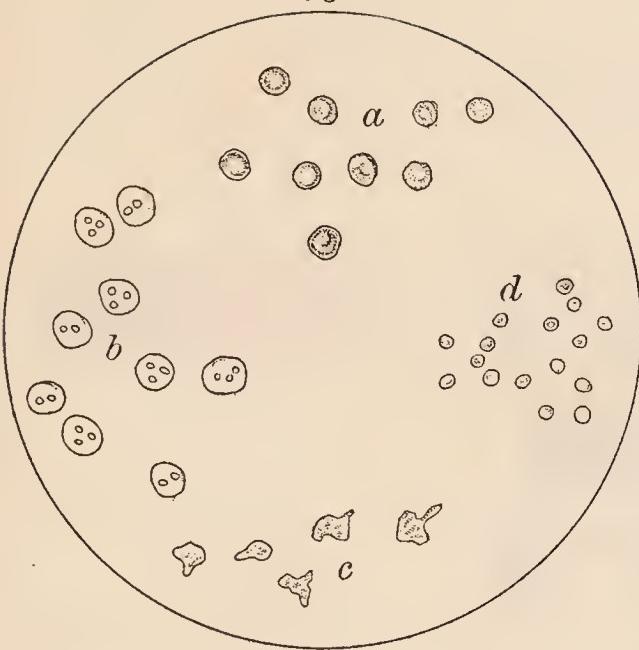


PLATE III.

13. *a*, Normal pus corpuscles or mucus corpuscles, granular.
b, Pus corpuscles swollen with acetic acid, showing nuclei.
c, Pus corpuscles showing ameboid movement. All colorless.
d, Blood corpuscles, nearly colorless.
14. Different forms of epithelial cells, colorless.
15. Granular casts, colorless.
16. Epithelial casts, colorless.
17. Hyaline casts.
 - a*, Broad or waxy, colorless.
 - b*, Narrow, colorless, and extremely transparent.
18. *a*, Fat-casts.
b, Yeast fungi in urine.
c, Spermatozoa.

PLATE III.

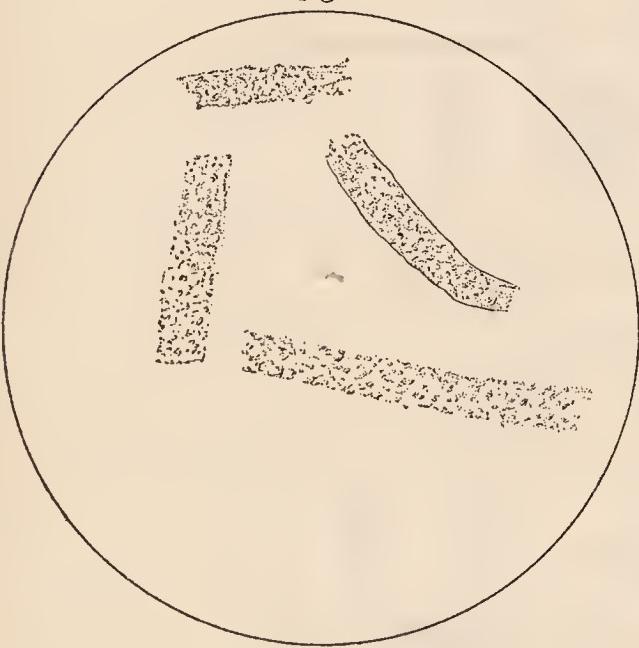
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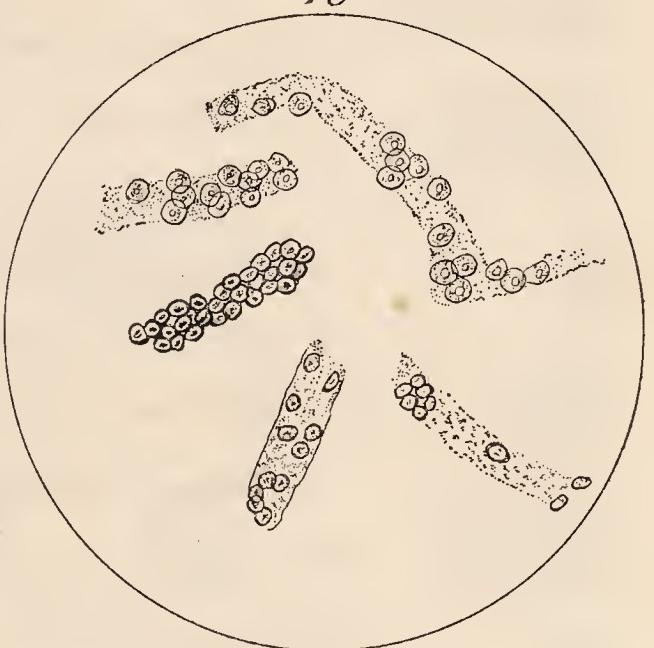
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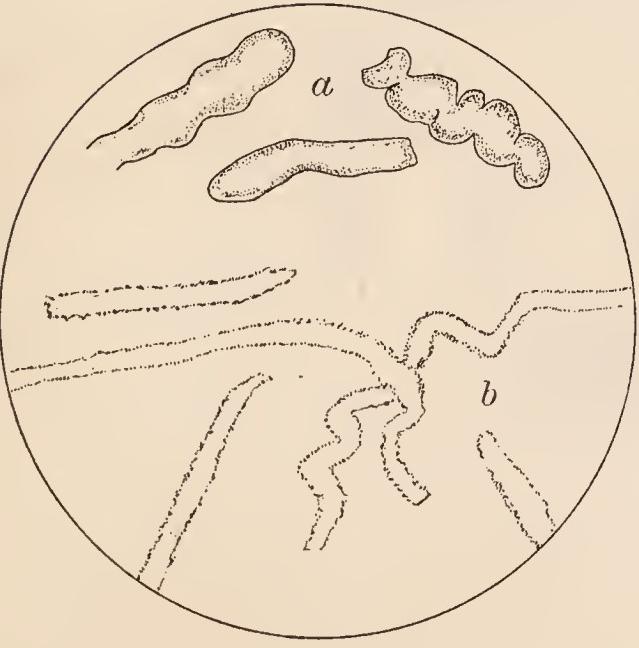
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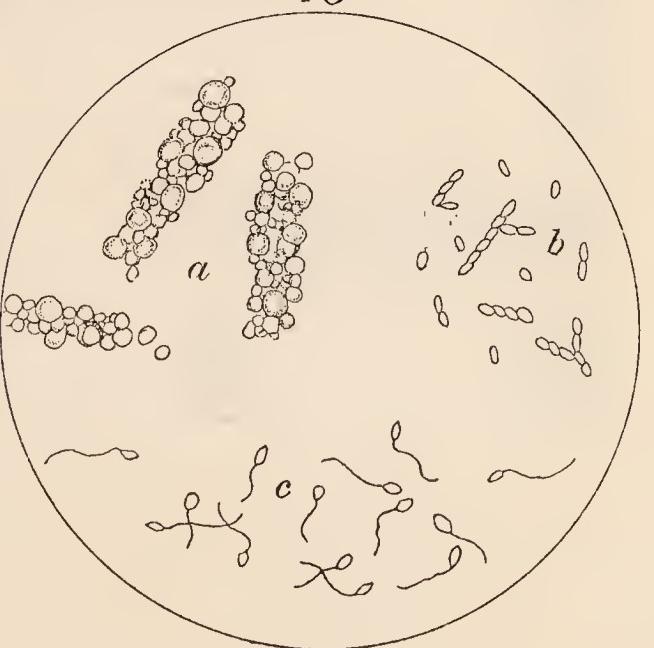
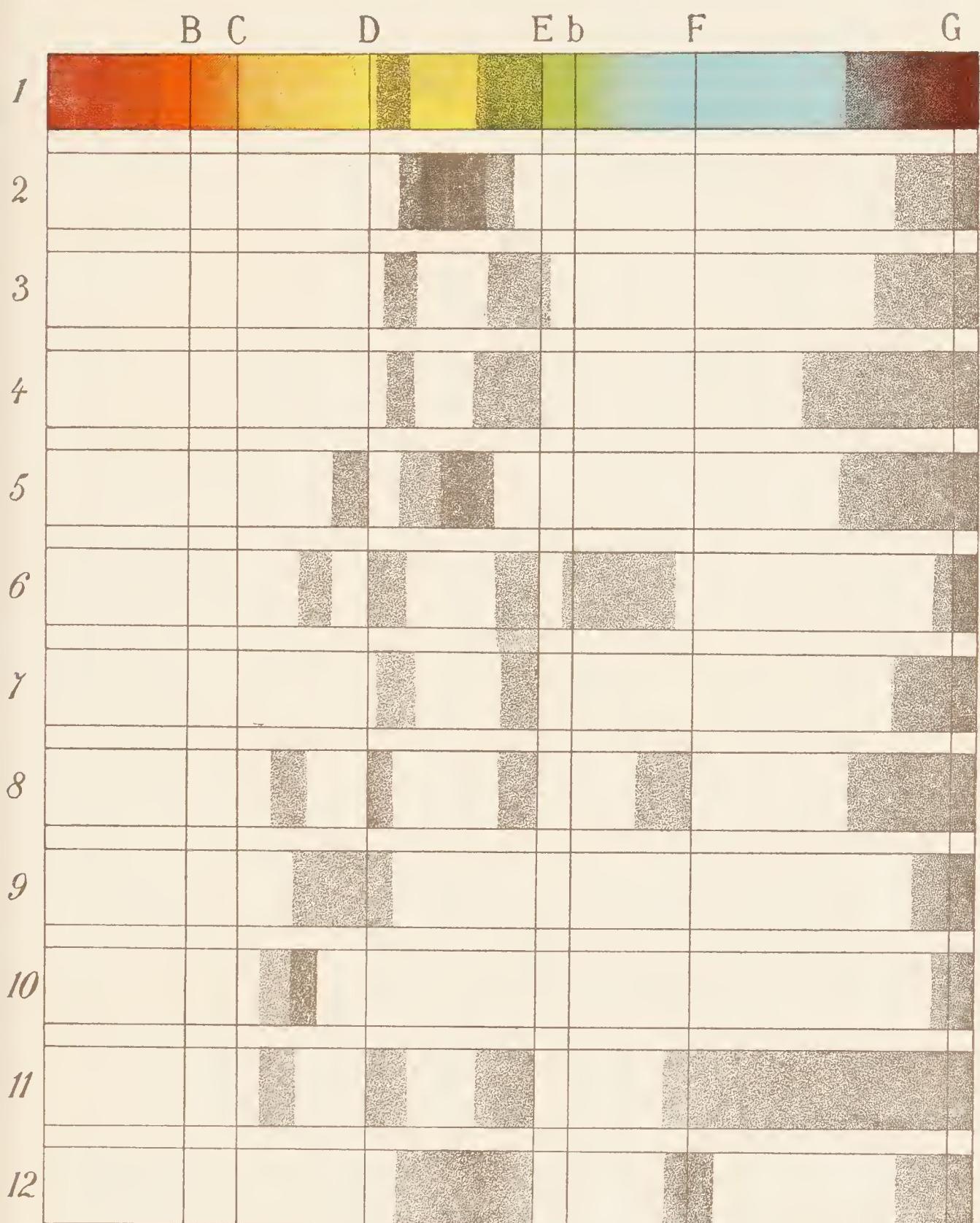


PLATE IV.

ABSORPTION SPECTRA.

1. Oxyhemoglobin.
2. Hemoglobin.
3. CO-hemoglobin and CO-hemochromogen.
4. Methemoglobin, alkaline.
5. Hematoporphyrin, acid.
6. Hematoporphyrin, alkaline.
7. Hemochromogen, alkaline.
8. Hematin, acid.
9. Hematin, alkaline.
10. Sulphur methemoglobin.
11. Methemoglobin, neutral or faintly acid.
12. Pettenkofer's test for biliary acids.

PLATE IV.



INTRODUCTION.

THE principal materials which enter into the composition of the animal body, as well as the food necessary for its support, may be divided into several general classes:—

- I. Inorganic { 1. Water.
 2. Mineral substances.
- II. Organic { 1. Non-nitrogenous { (a) Carbohydrates.
 compounds, such as { (b) Fats.
 2. Nitrogenous compounds, such as the
 Proteins.

There are a number of nitrogenous compounds in the animal body which cannot be classed under the proteins, and others which contain no nitrogen, but which do not belong to the carbohydrates or fats; nevertheless, these three classes include, by far, the largest part of the organic constituents.

THE CARBOHYDRATES.

The carbohydrates are composed of three elements: carbon, hydrogen, and oxygen. The latter two are always present in proportion to form water, and in this the carbohydrates differ from the fats, which contain less oxygen. The name of the group is derived from their composition, although they cannot be made directly from carbon and water. Most of them contain in a molecule six atoms of carbon or some multiple of six. Many organic compounds of carbon, hydrogen, and oxy-

gen, however, have the two latter in the proportion to form water, but do not belong to the carbohydrates.

Carbohydrates are found in both the animal and vegetable kingdoms, but are more abundant in the latter.

The different members of the group differ greatly in their properties, such as the power of crystallization, fermentation, reducing effect, action on polarized light, taste, etc.

They may be divided according to their molecular composition into three classes:—

I. Glucoses, or monosaccharids, $C_6H_{12}O_6$, including:

1. Glucose, or grape sugar; also called dextrose.
2. Fructose, or fruit sugar; also called levulose.
3. Less important are galactose, mannose, and others.

II. Saccharoses, or disaccharids, $C_{12}H_{22}O_{11}$, including:—

1. Sucrose, or cane sugar.
2. Lactose, or milk sugar.
3. Maltose, or malt sugar, and some others.

III. Amyloses, or polysaccharids, $(C_6H_{10}O_5)_x$, including:—

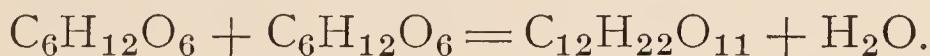
1. Starch.
2. Dextrin.
3. Glycogen.
4. Cellulose; also a number of gums and others of less importance.

In addition to the above classes there are a number of compounds containing three, four, five, seven, eight, or nine atoms of carbon in a molecule. Their chemical

names are compounded from a prefix which indicates the number of carbon atoms with the suffix *ose*, thus, triose, tetrose, pentose, etc. As yet they are not known to be of any great importance in physiological chemistry.

The first class, the monosaccharids, is so named because they contain one of the groups of six carbon atoms. They are mostly crystalline, easily soluble in water, and have a sweet taste. Chemically they are aldehyde or ketone compounds of the hexatomic alcohols. The former are indicated by the prefix *aldo-*, the latter by *keto-*; thus *aldo-pentose*, *keto-hexose*, etc. They have the power of reducing the oxygen compounds of the metals and of forming compounds with phenyl-hydrazine. They will also undergo fermentation with yeast.

The disaccharids contain in a molecule twelve atoms of carbon. They may be conceived of as composed of two molecules of a monosaccharid minus one molecule of water:



By the action of acids or ferments they take up a molecule of water and form two molecules of a monosaccharid. This formation of one or more simple sugars from a molecule of a disaccharid is called inversion, and the resulting sugar is known as invert sugar. The disaccharids do not undergo fermentation with yeast until they have been inverted. They are all soluble in water and have a sweet taste.

The polysaccharids contain more than two groups of six carbon atoms, though the number is, in most cases, not positively known, and therefore is represented by *x*. They have probably a much higher molecular weight than the other classes. Their constitution is not known.

They are mostly amorphous, and, except the dextrins, insoluble in water or soluble with difficulty, consequently nearly or quite tasteless. Ferments and acids in the presence of water convert them by hydrolysis into the monosaccharids. They usually do not have reducing power.

STARCH.

Starch occurs in the cells of the plant. It is in the form of grains or granules, which vary in size in different plants from about 0.002 of a millimeter to ten times that. The granules are composed of two parts: an inner, soluble one, called granulose, and an outer one, called cellulose. This latter is insoluble in water and protects the starch from the action of many of the weaker ferments. When boiled or acted upon by alkalies it is broken, allowing the granulose to escape and forming starch-paste, or soluble starch. The shape and size of the granules differ so much in the different plants that the source can often be determined by its microscopic appearance. Those of the potato have a shape somewhat similar to that of a clam shell, those of wheat are round and smaller, and those of buckwheat more irregular. (Plate I, 1 and 2.)

Starch can be obtained from the parts of the plant where it is stored up, like the tuber of the potato or the kernel of grain, by macerating it, then washing out the starch with cold water.

Starch is a colloid. A colloid is a substance which when dissolved will not pass through an animal membrane or parchment. Colloids are the opposite of crystalloids, which are usually crystalline and which will diffuse through such membranes. This process of diffusion or

separation of colloid from crystalloid substances is called dialysis. As starch cannot pass through an animal membrane, it must be changed to a diffusible form before it can be absorbed. This is effected by ferment in the saliva and pancreatic fluid.

By heating to 160° to 200° starch is converted into dextrin.¹ By boiling a solution with a dilute acid it is changed first into dextrin, then into glucose. Ptyalin changes it first into dextrin, finally to maltose. The diastase of malt gives the same products.

Starch gives an intense blue color with a solution of iodine. This color disappears on heating the liquid; but if it is not heated too long it becomes blue or purple again when it cools. The color will also be destroyed by the addition of anything which will form a compound with the iodine, such as sodium thiosulphate, silver salts, or the alkaline hydroxides. Enough starch for the microscope examination can be obtained from the scrapings from a potato without washing. The cellulose fibers will then be seen also.

1. Starch may be prepared from a potato by grating it upon a tin grater, stirring it up with a little water, and squeezing the water, which contains a large part of the starch, through a piece of unbleached muslin. After repeating this with several portions collect the water in one vessel and allow the starch to settle to the bottom. Pour off the water, add more, and allow to settle again, repeating till the starch appears clean and white. Take what is needed for the experiments and let the rest dry.

2. Examine the starch under the microscope. Notice the shape of the granules.

3. Place a drop of very dilute iodine solution upon

¹ Unless otherwise stated, all degrees of temperature will be understood as referring to the centigrade scale.

the slide so that it runs under the cover glass and notice the markings which are thus brought out upon the granules which are least colored. Sketch these.

4. Why does the iodine stain bring out these more distinctly?

5. Examine in the same way starch from other sources: corn, wheat, buckwheat, etc. Observe the difference in the size and shape of the granules.

Sketch these and hand in the results.

6. Get specimens of unknown starches from instructor and determine source microscopically.

7. Prove that starch does not dissolve in cold water by filtering after shaking powdered starch in a test tube of water. Iodine gives no color to the filtrate.

8. Add about a gram of starch to 100 c.c. of cold water, mix it thoroughly, and boil. The starch is dissolved, as is shown by filtering and, after cooling, testing a portion of the filtrate with an iodine solution. A deep blue color is produced. It is destroyed by heating, but reappears as purple or blue again upon cooling.

9. What is the explanation of the variation of color with temperature?

10. Use a piece of parchment dialyzing tube to test diffusibility. First see that this does not leak. It should hold water when suspended by the two ends. Place inside some of the starch solution made in the preceding experiment and hang the whole in a small beaker of water, so that the liquids inside and outside are at the same level. Instead of the tube a piece of parchment can be placed in a funnel from which the stem has been broken, as if the liquid were to be filtered. Pour the starch solution into this and suspend the whole in a

beaker of water. Allow it to stand several hours, then test the water outside with iodine for starch. It does not pass through because it is a colloid. Then put a little glucose in the dialyzer. It diffuses out and can be found by Trommer's reaction (40).

11. Why does one carbohydrate pass through the membrane while the other does not?

12. Examine under the microscope the starch paste which has been made by heating starch in water. The granules have been burst open and destroyed.

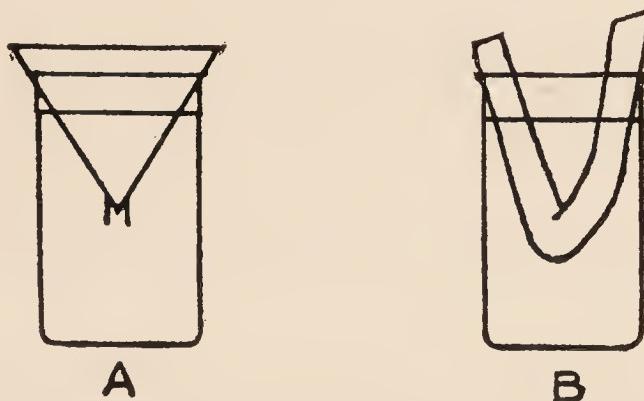


Fig. 1.—Two forms of simple dialyzers. *A* is a funnel with a parchment paper supported so that the water outside is at the same level as the liquid inside. *B* is a tubing of parchment paper open at the ends, the water and diffusing liquid being at the same level.

13. Prove that starch can be hydrolyzed by acids as follows:

In about 100 c.c. of water in a porcelain dish boil enough starch, previously moistened with cold water, to make a thin paste. Add about 10 c.c. of dilute sulphuric acid and boil, stirring at first until the liquid becomes thinner. Keep the solution up to its original volume by the addition of water. If this is not done the strong acid will turn the liquid brown or black. From time to time remove a portion, cool, and test with iodine. When

the iodine gives a red color the starch has been converted into dextrin. When no color appears on the addition of iodine it has been changed to glucose. Test a portion for glucose by adding an equal volume of sodium hydroxide solution, then, drop by drop, cupric sulphate solution till a deep blue color is produced. Heating this will give a yellow or red precipitate, showing the presence of glucose. This is known as Trommer's test for glucose (40).

14. What is hydrolysis? What is composition of yellow precipitate? Of the red one? Why the difference? Equation for action of sodium hydroxide on cupric sulphate? For heating the product? Explain the production of a purple color by the iodine. How is the reducing power of glucose related to its molecular structure?

15. Try Trommer's test (40) with the starch solution. It does not respond.

16. Add gradually to the remainder of the solution which has been boiled with the acid, while it is still hot, powdered calcium carbonate until it is neutral. Filter and evaporate the filtrate to dryness on the steam bath.¹ Glucose remains: examine its properties and preserve it for subsequent tests.

17. What becomes of the acid? Equation?

DEXTRIN.

Dextrin is the intermediate product in the change from starch to maltose or glucose. There have been

¹ To evaporate a liquid on a steam or water bath the evaporating dish in which it is contained can be heated by standing it on a beaker of boiling water. This removes all danger of burning the residue.

several varieties described: erythrodextrin, which is colored red by iodine; achroödextrin, which is not so colored, etc.

It is formed from starch by the action of heat, acids, or ferments. It is soluble in water, making a sticky liquid, often used for a mucilage. It is produced when bread is toasted, and is also found in the crust. Toast or bread crust, then, has its starch partially changed into a more diffusible substance.

18. Prepare dextrin from starch by heating in a porcelain dish on a sand bath half a spoonful of powdered starch. The starch must be stirred with a glass rod until it has turned yellowish or brown, when it has been changed to dextrin.

19. Dissolve some dextrin in water and test with a drop of iodine solution. A red or brown color is produced, not a blue, if the change has been complete. If commercial dextrin is tested it will probably be found to contain undecomposed starch.

20. Why? How would such a mixture be indicated? Why?

21. Toast a small piece of wheat cracker or bread over the flame until it is brown; extract with a little water, and test the filtrate for dextrin with a few drops of iodine solution. If some unchanged starch remains compare the color with that obtained from an extract of the untoasted cracker or bread. Would the digestibility of starchy foods be modified by this process? Why?

GLYCOGEN.

Glycogen is found in a few of the lower plants, in some shellfish, and in many fluids and tissues of the bodies of mammals. It is most abundant in the liver, and next in the muscles. It is also called liver sugar or liver

starch. In the animal body it is most plentiful when the animal is well nourished, especially after a full meal. At such times it may be in as large an amount as 10 or 12 per cent. of the liver, but it is usually not more than 3 or 4 per cent. It disappears completely from the liver after long starvation, or more quickly through severe work or great fright.

It is best obtained from the liver. After boiling to kill the ferments which are always present, dissolving in water, and removing the nitrogenous substances, it can be precipitated by alcohol.

Glycogen is an amorphous, white, tasteless powder. In water it dissolves to an opalescent solution. With iodine it gives a red color, which disappears on heating. It does not have a reducing action upon cupric hydroxide. Boiling with acids converts it into dextrin, then maltose, then glucose. The salivary and pancreatic ferments produce the same change.

The glycogen of the liver seems to be formed mostly from the carbohydrates of the food, but partly, at least, from the nitrogenous compounds.

It is deposited in the liver as a reserve material, just as the starch is stored for a reserve material in the plants. When it is needed by the body it is converted by a ferment into dextrose, and this passes into the circulation. It is probable that it is used to furnish energy for the body. After death the glycogen quickly disappears from the tissues of the body, being decomposed by the ferments which are present. If these are destroyed by boiling the tissue for a short time the glycogen is not destroyed, but can be extracted.

22. Preparation of Glycogen.—In a mortar grind with sand or glass about 25 grams of the adductor muscle of the scallop

(pecten irradians), extract several times with 50 c.c. of cold water, repeating the operation with hot water. Boil the liquid to coagulate the proteins, filter and concentrate the filtrate to about 50 c.c., then add alcohol to 70 per cent. in concentration. This precipitates the glycogen. Filter this out. If the dry powder is desired, wash with alcohol, then with ether, and dry in a desiccator.

23. Prepare glycogen from the liver of a freshly killed, well-nourished animal. The animal is best killed while digestion is in progress. If a rabbit, this may be an hour after introducing 10 to 15 grams of sugar into the stomach through a tube. Remove the liver as soon as possible, cut it into lumps, and immediately put it into about four times its weight of boiling water. Let it boil half an hour, then rub up the lumps as much as possible in a large mortar, add water, and boil again. Filter through muslin, concentrate upon the water bath to about one-fourth its volume, and allow the solution to cool. Then precipitate the gelatin and other protein compounds by adding alternately small quantities of hydrochloric acid and potassium-mercuric iodide¹ as long as anything is thrown down. Filter and add to the filtrate twice its volume of alcohol to precipitate the glycogen. Wash with alcohol. To purify the substance it should be dissolved in a little water and precipitated again with alcohol. If the anhydrous powder is desired, the water must be removed as far as possible before drying. To accomplish this wash the precipitate next with absolute alcohol, then with ether to remove the alcohol. Dry in a vacuum desiccator over sulphuric acid. If the pure substance is not desired, the tests may be made on the solution after the removal of the protein compounds.

24. If the dry substance has been obtained, try its taste and its solubility in water. Test the solution with iodine. It gives a red color.

25. Try Trommer's test (40). There is no red color

¹ Prepare by precipitating mercuric chloride with potassium iodide, washing the precipitate and then adding it to a hot solution of potassium iodide as long as it dissolves.

if the glycogen has been purified. If it has not been it contains glucose, which responds to the test.

26. Convert one portion of the solution into glucose by heating with hydrochloric acid and another by the action of saliva. Test each for the glucose by Trommer's test (40).

27. Equation for action of acid? How does that for action of saliva compare with it?

28. Prove that the glycogen is destroyed (changed to a reducing sugar) in the liver after death by the action of a ferment, making the test upon some liver from the market. (Instead of this a part of the liver from 23 can be used. This should be after it has stood several hours in a warm place.) Chop it finely and extract with boiling water. Acidify the solution slightly with acetic acid, add a little sodium chloride, and boil to precipitate the protein compounds. After filtering, test the filtrate for glycogen by means of iodine and also for sugar by Trommer's test (40).

29. Add a little blood to a test tube of the glycogen solution and, after it has stood ten minutes in a beaker of water at body temperature, slightly acidify with acetic acid, boil, and filter to remove the albumin, and test the filtrate for glucose and glycogen. The latter has been converted into glucose by a ferment which is found in the blood.

CELLULOSE.

Cellulose forms the wall of the plant cells, and is not found as a constituent of the animal body, except in a few of the lower forms. Cotton and filter paper are two of the most common examples. It is distinguished from

the other polysaccharids by its insolubility. It is insoluble in the ordinary solvents, but can be dissolved in the strong mineral acids, being converted into dextrin. It also dissolves in a solution of cupric hydroxide in ammonia (Schweitzer's reagent), and in a solution of zinc chloride (Schultze's reagent). Sulphuric acid changes paper into a parchment-like substance by covering the surface with a coating of its decomposition products and so sticking the fibers together. Iodine does not stain the unaltered cellulose, but does so after it has been acted upon by the acid. Cellulose is only slightly attacked by the digestive ferments of man, though the herbivorous animals digest it to a greater extent. By the continued action of acids it is converted into glucose.

30. Show that cellulose is not stained by iodine. Use absorbent cotton or starch free filter paper.

31. Try the solubility of cotton or filter paper in solution of zinc chloride in concentrated hydrochloric acid (Schultze's reagent) and also in a solution of cupric hydroxide in ammonium hydroxide (Schweitzer's reagent). It can be precipitated from these solutions by dilution with water.

32. To one volume of water in a beaker add slowly two volumes of concentrated sulphuric acid, stirring meanwhile. Cool the mixture; then immerse in it for a few seconds a piece of heavy filter paper, plunging it into a large beaker of cold water as soon as it is removed. If the time of immersion has been correct it will be semi-transparent after washing, and as tough as an animal membrane. It is called vegetable parchment. It can be stained blue by iodine. Amyloid has been formed.

33. Let another piece of paper remain in a small amount of the warm acid of last experiment until it has

entirely disappeared. Then dilute a little of the acid with water and test it for glucose by Trommer's test (40), being sure that enough alkali has been added to give it an alkaline reaction.

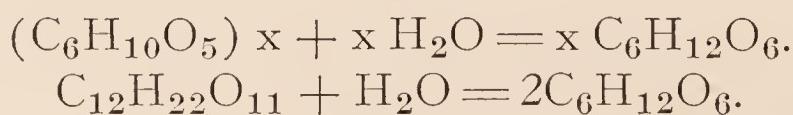
34. How does this action compare with that of concentrated sulphuric acid on cellulose? Equations for both?

35. Make a table showing comparative properties of the polysaccharids, as shown by your experiments.

GLUCOSE ($C_6H_{12}O_6$).

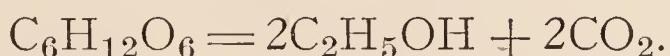
Glucose is also called dextrose and grape sugar. It is found in the vegetable kingdom as well as in the animal. It is normally present in the blood and lymph and in other fluids of the body. Pathologically it is found in considerable quantities in the urine, sometimes in as large amounts as 10 per cent. or more. The urine may also temporarily contain dextrose after a diet rich in carbohydrates. In the urine of a normal individual it can be continually present only in a minute amount, too small to be detected by the ordinary tests.

Glucose is made commercially by boiling starch with a dilute acid. It can be produced from any of the polysaccharids or disaccharids in the same manner. They unite with one or more molecules of water, forming glucose:—



Pure glucose can be made from pure cane sugar by dissolving it in alcohol and adding hydrochloric acid. The glucose crystallizes out on standing and can be purified by washing and recrystallization.

Glucose is a crystalline substance, but crystallizes with difficulty from water. It can be better crystallized from methyl alcohol or ethyl alcohol. Its taste is sweet, but less so than that of cane sugar. It is easily soluble in water or hot alcohol. With yeast, glucose ferments best at about 25° C., forming alcohol and carbon dioxide:—



In the presence of milk or cheese it ferments to lactic acid. Calcium carbonate or oxide of zinc can be added to keep the solution neutral if it is desired that the action go on for a long time, as the presence of the acid destroys the ferment:—



By the action of another ferment the lactic acid is changed into butyric acid:—



Dextrose, like many other organic compounds, has the power to change the direction of the vibrations in a ray of light. With ordinary light these vibrations are at right angles to the direction of the ray, but in no single plane. By passing the ray through a Nicol prism of Iceland spar all vibrations are cut out except those of a single plane. The light is then said to be polarized. The polariscope is an instrument which has two of these Nicol prisms; if they are turned at the same angle and a ray of light strikes them successively it passes through both. If one is turned at an angle of 90° to the other no light passes, since the second cuts off the light which passed the first.

In the polariscope the solution to be examined is placed in a horizontal tube and the ends of the tube are closed by glass plates; the tube lies between the two Nicol prisms. Let us suppose that before placing the tube in the polariscope the two prisms are rotated to the same angle so that a ray of light passes through both, and let us suppose, furthermore, that the vibrations of the ray are vertical while the ray is, as a whole, moving in a horizontal direction. Now, if a sugar solution in a tube is inserted and if it twists, or rotates, the direction of

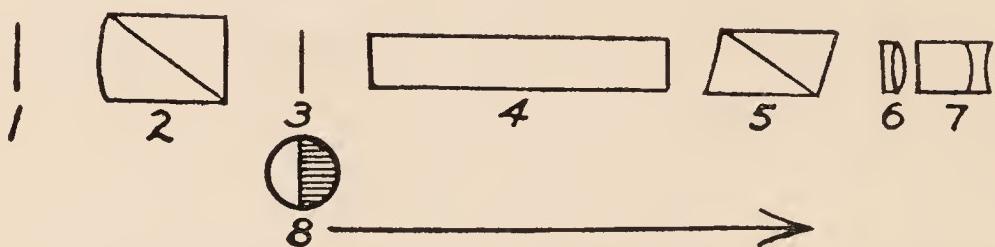


Fig. 2.—Representing passage of light through Laurent polariscope. 1, yellow plate to obtain monochromatic light; 2, first Nicol prism (the polarizer); 3, quartz plate covering half the field; 4, tube containing the solution for testing; 5, second Nicol prism (the analyzer); 6, and 7, ocular for focusing the image; 8, shows the two halves of the field.

vibrations of the light rays away from the vertical, it is evident that the ray cannot pass the second Nicol prism because this is set so that only the vertical vibrations can pass. If the second Nicol prism is rotated so that the light does pass, the number of degrees through which it must be revolved indicates the rotatory power of the substance which is being tested.

The specific rotation of a substance is represented by the rotation from a solution of 1 gram per c.c. in a tube 10 cm. long. Sodium light must be used. The specific rotation can be calculated from the observation as follows:

$$\text{Specific rotation} = \frac{\text{observed rotation}}{\text{length of tube in dms.}^* \times \text{gram per c.c. of solution}}$$

The specific rotation of dextrose is 52.8° .

If the specific rotation of a substance is known, by transposing the above formula the per cent. in solution can be determined.

$$\text{Percentage} = \frac{\text{observed rotation} \times 100}{\text{specific rotation} \times \text{length of tube in dms.}}$$

" 36. What would be the observed rotation of a 10 per cent. solution of dextrose using a tube 5 cm. in length?

If a solution of dextrose of unknown concentration gave an observed rotation of 10° using a tube 10 cm. in length, what would be its percentage of sugar?

37. Prepare pure glucose from cane sugar by the following method:

Acidify 100 c.c. of 90 per cent. alcohol with 4 c.c. of concentrated hydrochloric acid, warm the liquid upon the water bath to 45° , and add gradually 30 grams of finely powdered cane sugar, stirring until it has dissolved. The temperature should not rise above 50° . After two hours at 50° the sucrose has been inverted. Then let it stand in a cool place. The glucose commences to crystallize out in about a week, but crystallization may be hastened by adding to the cold solution a few crystals of glucose and by frequent stirring. After the glucose has crystallized from the solution filter, best with the aid of a filter-pump; wash free from the acid by 90 per cent. alcohol, then by absolute alcohol; finally dry the crystals. It may be purified by dissolving in pure methyl alcohol by the aid of heat and allowing it to again crystallize out.

37a. Test a dextrose solution with iodine and notice that the result is negative.

* dm. = decimeter.

Reactions of Dextrose which can be Used to Test for its Presence.

38. **The Alkali Test** (Moore's test).—Make the glucose solution alkaline with sodium hydroxide and boil for several minutes. The liquid turns yellow; as a test for glucose it is not very satisfactory.

39. **The Alpha-naphthol Test** (Molisch's test).—Add to about 5 c.c. of the sugar solution 2 to 3 drops of the reagent (a 15 per cent. alcoholic solution of alpha naphthol), then pour this slowly on to an equal volume of concentrated sulphuric acid, holding the tube in a slanting manner, so that the two do not mix. A reddish violet zone appears at the junction of the two liquids. This is a carbohydrate test, not a specific test for dextrose.

Reduction Tests.—From the ease with which dextrose can be oxidized in solution there have been developed many tests in which the monosaccharid removes oxygen from various metallic compounds, reducing them to the metals or to some lower oxide. These are called reduction tests.

40. **The Cupric Hydroxide Test** (Trommer's test).—Prove that cupric hydroxide (made by the addition of a few drops of copper sulphate solution to a sodium hydroxide solution) is soluble in a solution of glucose, in presence of an excess of sodium hydroxide, giving a deep blue liquid.

Show that this blue solution of copper and sugar is decomposed by heating and yellow or red precipitate of cuprous oxide is produced.

Prove that when heated alone in water cupric hydrox-

ide yields the black cupric oxide, not the red cuprous oxide, and that a large excess of the copper solution with a very small amount of glucose may cause the black to hide the red precipitate.

To perform Trommer's test, mix about equal volumes of sodium hydroxide and the glucose solution, then, while shaking, drop in copper sulphate solution slowly until a permanent precipitate begins to form, or until the liquid is deep blue; finally heat the solution.

41. Equations for reactions?

42. **The Copper Hydroxide-Rochelle Salt Test (Fehling's test).** Show that cupric hydroxide is soluble in a solution of Rochelle salt (potassium sodium tartrate) with sodium hydroxide, giving a deep blue liquid which is not decomposed by boiling.

Add to this a little glucose and heat; the reddish yellow Cu_2O is precipitated as in Trommer's test.

43. What is the function of the Rochelle salt? What is its composition?

44. **The Cupric Hydroxide-Glycerol Test (Haines' test).** Precipitate the cupric hydroxide from copper sulphate with sodium hydroxide, show that it is soluble in glycerol and that this solution does not decompose on boiling, then add a little glucose and show that the red cuprous oxide is precipitated on boiling just as in Fehling's test.

45. What is the function of the glycerol? How does Haines' test differ from Trommer's? From Fehling's?

46. **Benedict's Qualitative Test.**—The reagent contains:—

Crystallized copper sulphate.....	17.5 grams
Sodium citrate	173.0 grams
Sodium carbonate, dry.....	100.0 grams

Dissolve the citrate and carbonate in about 600 c.c. of hot water, and make up to 850 c.c. Dissolve the copper salt in water and make up to 150 c.c. Slowly pour, with constant stirring, the second solution into the first.

This reagent differs from Benedict's quantitative reagent.

To 5 c.c. of the reagent in a test tube add a few drops of glucose solution; boil for 1 to 2 minutes. A greenish, yellow or red precipitate appears, depending upon the amount of sugar present.

47. What is the function of the citrate? What other substances have a similar action?

48. **Copper Phosphate Test** (Folin and McEllroy).—The reagent is prepared by dissolving 13 grams of copper sulphate in about 200 c.c. of water and adding this to a solution containing in about 1 liter of water 100 grams of sodium pyrophosphate U. S. P., 30 grams of crystallized disodium phosphate and 50 grams of dry sodium carbonate.

Take 5 c.c. of the reagent in a test tube, add 5 to 8 drops of solution to be tested for sugar and boil one minute, or let the tube stand three to five minutes in boiling water. Small amounts of sugar cause a marked turbidity, larger ones give the cuprous oxide precipitate. A slight turbidity appearing only after cooling may be given by other reducing substances.

49. **The Copper Acetate Test** (Barfoed's test).—To about 5 c.c. of Barfoed's reagent (containing in 100 c.c. of water 4.5 grams of crystallized copper acetate and 0.6 c.c. of glacial acetic acid) add 1 to 2 c.c. of dextrose solution in a test tube and let it stand for five to ten minutes in a beaker of boiling water. Red cuprous oxide is formed, but more slowly than with Trommer's reagent.

50. Comparing Trommer's and Barfoed's reagents, which should be the more readily reduced, and why?

51. **The Bismuth Subnitrate Test (Böttger's test).**—Show that glucose will reduce the subnitrate, or basic nitrate, of bismuth if its solution is made alkaline with sodium hydroxide or carbonate and boiled with the bismuth compound. Metallic bismuth is precipitated as a black powder, but if there is an excess of the subnitrate the mixture may appear gray.

52. **The Bismuth-Rochelle Salt Test (Almen-Nylander test).**—Prove that bismuth subnitrate is soluble in an alkaline solution of Rochelle salt, similarly to cupric hydroxide. Take about half a gram of Rochelle salt with a gram of the subnitrate and heat it for some time with 20 to 25 c.c. of 10 per cent. sodium hydroxide. If all does not dissolve, the clear liquid can be filtered or decanted off. Heat this with a little glucose, and compare result with that of Böttger's test.

53. Try the relative sensitiveness of tests, using a 0.1 per cent. to 0.2 per cent. solution of dextrose, and tests selected by instructor. Report conclusions.

54. **The Phenylhydrazine Test.**—In a test tube mix 3 to 5 c.c. of dextrose solution with 1 c.c. (not more) of a phenylhydrazine solution. (This is a mixture of equal volumes of 50 per cent. acetic acid and phenylhydrazine, the base). Place the tube in a beaker of boiling water and heat it from half an hour to one hour. Then cool it slowly and examine the precipitate with the microscope. It is phenyl-glucosazone: bright yellow, needle shaped crystals. They may be single, but are more often in clusters (Plate I, 5). They can be distinguished, if necessary, from similar compounds of the other sugars by their melting point, which is 204° C.

The formation of glucosazone crystals is hindered or entirely prevented if much unchanged glucose remains in the solution, consequently it is often better to use a very small volume of the sugar solution in order to avoid this excess of sugar.

55. Sketch crystals in note-book. Is this a reduction reaction?
56. Crush a piece of condensed yeast as large as a pea in a test tube completely full of a glucose solution. Mix and place it, still full of the liquid, with the mouth downward in a beaker which contains a little water, or better, some of the grape sugar solution. Let it stand for twenty-four hours in a warm place. The carbon dioxide which is formed is found in the test tube and the alcohol in the liquid. The gas may be proved to be carbon dioxide by shaking it with lime water, which it turns white. The presence of the alcohol is shown by warming the liquid after the addition of iodine and a little sodium hydroxide. Iodoform separates out in yellow scales, or, if the amount of alcohol is very small, the odor alone may be perceived.

57. Equation for reaction? How does the change compare with the phenylhydrazine and reduction tests? What is the formula for iodoform? Sketch the crystals.

It should be borne in mind that no one reaction is sufficient to prove the presence of dextrose. Molisch's test depends upon the formation of furfural, which is made by the action of acids upon any carbohydrate, free or combined. Phenylhydrazine reacts with aldehydes or ketones, and thus forms osazones with other sugars. The melting points of these differ and are characteristic, though the shapes of the crystals are very similar. The reducing power of glucose comes from its aldehyde radical; therefore, sugars which contain this will reduce. In the body fluids are other compounds which do the same. Yeast also will ferment some other sugars, although they are not usually found in the body tissues.

Quantitative Tests for Glucose.

58. Fehling's Method.—The solutions used are: (A) 34.64 grams of cupric sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), dissolved in enough water to make the volume 500 c.c. The crystals used must be dark blue and not effloresced; (B) 187 grams of pure Rochelle salt and 68 grams of sodium hydroxide in water enough to make the volume 500 c.c. These solutions must be kept separate.

For each determination mix 5 c.c. of *A* with 5 c.c. of *B*, measuring carefully with a pipette. Add about 40 c.c. of water, and heat to boiling in a beaker or porcelain dish. If the reagent is good there will be no red precipitate.

The best results are obtained when the solution contains from 0.5 per cent. to 1.0 per cent. of sugar; that is, when from 5 to 10 c.c. are necessary to destroy the blue color of the Fehling solution. If it contains more than this, it must be diluted with water to 5 or 10 times its volume, measuring accurately the water added and mixing thoroughly.

The Fehling solution after dilution is heated to boiling, and the sugar solution run in from a burette until the blue color has been destroyed, leaving the liquid colorless above the red precipitate. If too much sugar has been added it begins to turn yellowish. The amount of sugar is ascertained most quickly by making two determinations: first, a rough one, then one which is made more carefully. Make the first by running in the sugar solution, 2 or 3 c.c. at a time, as long as the blue color is well marked, then 1 c.c. at a time, heating to boiling after each addition. It can be learned by this first test within 1 or 2 c.c. how much will be required. Then rinse out the

beaker, take again 10 c.c. of the Fehling solution, diluted as before; heat to boiling and run in at once within 1 c.c. of the necessary amount of the sugar solution. Bring it to a boil. Then add the sugar solution a few drops at a time, heating after each addition, until the blue color has just been decolorized, the red color of the precipitate being disregarded.

There are thus three checks on the accuracy of the titration—before enough sugar has been run in the liquid is blue; with the correct amount it is perfectly colorless; with too much sugar it becomes yellow.

Since 10 c.c. of the Fehling solution is decolorized by 0.05 gram of glucose, the amount of the sugar solution or urine which has been used from the burette must have contained 0.05 gram of glucose. Read the volume which has been poured from the burette, and calculate the percentage of sugar in the original solution. If this has been diluted with water the amount in the dilute solution must be multiplied by the number of times it was diluted. Remember that however much of the sugar solution may have been used to destroy the blue color, it contained 0.05 gram of sugar. For example, if the amount used was 10 c.c., there would be 0.005 gram of glucose in 1 c.c.; that is, in about 1 gram of solution. In 100 grams there would be approximately 0.5 gram of glucose, or 0.5 per cent.

59. Benedict's Method.—This is a modification of Fehling's, the reagent containing ferrocyanide and thiocyanate, whereby the red cuprous oxide is prevented from precipitating. The reduced copper is thrown down as white cuprous thiocyanate, the end point being to many analysts more distinct than with Fehling's solution. The reagent also has the advantage of being stable.

Benedict's quantitative reagent contains:

Copper sulphate, crystallized, CuSO ₄ , 5H ₂ O...	18 grams
Sodium carbonate, anhydrous.....	100 grams
(or twice as much of the crystals)	
Sodium citrate	200 grams
Sodium thiocyanate	125 grams
Potassium ferrocyanide (5 per cent. solution)	5 c.c.
Distilled water to make 1000 c.c.	

Dissolve the carbonate, citrate and thiocyanate in about 800 c.c. of hot water. Dissolve the copper sulphate in about 100 c.c. of water, and slowly add it to the first solution with constant stirring.¹ Add the ferrocyanide, cool, and make up to exactly 1000 c.c.

To determine the amount of sugar, measure with a pipette 25 c.c. of the reagent into a porcelain dish, add 5 to 10 grams of dry sodium carbonate and heat to boiling. While the solution is boiling, run in the sugar solution from a burette. This can be done rapidly at first, while the white precipitate is forming, but when the blue color grows faint only a few drops of the sugar solution should be added at a time. The boiling must be continuous, and if the volume is thereby decreased, water should be added. By regulating properly the speed with which the sugar solution is added this should be unnecessary. The disappearance of the blue color is the end point.

25 c.c of the reagent are reduced by 50 mg. of dextrose. Calculate the weight of sugar in 100 c.c. of the solution, and in the original solution if it was diluted.

60. Folin and McEllroy's Method.¹—The reagents are two,—a solution of copper sulphate and a dry mixture. The copper sulphate solution contains 60 grams of the

¹ Journal of Biological Chemistry, 1918, XXXIII, 513.

crystallized salt, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, in one liter. The dry mixture is made by mixing in a mortar 100 grams of disodium phosphate crystals, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 60 grams of dry sodium carbonate and 30 grams of sodium thiocyanate. 5 c.c. of the solution is reduced by 25 mg. of dextrose or 40.4 mg. of lactose.

The dextrose solution is run into the reagents from a burette, and the titration is carried out in a test tube. The end of the reaction is reached with the disappearance of the blue color. The phosphate and thiocyanate prevent the precipitation of cuprous oxide. If the percentage of sugar is low, so that a considerable volume of urine is necessary, the burette is read in the usual manner. If much sugar is present, so that it takes but 1 or 2 c.c. of urine, the volume used can be best determined by the *drop method* as follows:—

Fill the burette with the sugar solution (best by suction through a glass-tipped rubber tube and stopper in the top). In a test tube place a small pebble, to aid quiet boiling, 5 c.c. of the copper sulphate solution and 4 to 5 grams of the dry mixture. Heat until a clear solution is obtained. Add 25 drops of the sugar solution and boil *very gently* for two minutes. If all the copper is reduced more than 5 per cent. of dextrose is present, and a new determination should be made. If the copper is only partly reduced, add more of the dextrose solution, from 10 to 25 drops, depending upon the amount of un-reduced copper remaining; boil *gently* another minute. Keep account of the number of drops, boiling a minute after each addition, until the blue color has just disappeared. Then run in drops enough to make the total volume used equal to 1 c.c. or 2 c.c. Calculate from the number of drops the fraction of a c.c. used. For exam-

ple, if 37 drops were necessary to decolorize the reagent and 15 more to make a total volume of 1 c.c., or 52 in all, $\frac{37}{52}$ c.c. was the volume corresponding to 5 c.c. of the reagent, or 5 c.c. contained 25 mg. of dextrose.

With a small burette graduated to 0.02 c.c. and provided with an accessory tip drawn out to such a fine opening that it delivers 45 to 55 drops per c.c., small volumes can be measured without counting the drops. It should be filled from below by suction.

61. Determination of Percentage of Glucose in Solution by the Polarimeter (Demonstration by instructor).

The sugar solution must be colorless. If it is not, shake with a pinch of basic lead acetate, and filter through a dry filter. After rinsing the tube with the sugar solution, fill it, sliding the glass disk on so that there are no air bubbles, then screw on the cap. Place the tube in the polariscope, and, with the sodium flame, focus the eye-piece so that the field is sharp. Rotate the graduated disk so that the two fields are equally illuminated, then read the angle of rotation in degrees and hundredths by means of the vernier. Make several independent readings until they check. Notice whether the rotation was toward the right (dextro-rotatory) or toward the left (levo-rotatory). Calculate the percentage of glucose by the formula given above.

62. Determine concentration of unknown dextrose solutions by method selected by instructor.

LACTOSE (MILK SUGAR: $C_{12}H_{22}O_{11}+H_2O$).

Lactose is found in the milk of all mammals and occasionally during pregnancy in the urine. It can be obtained from the milk by crystallization after the removal of the nitrogenous constituents.

It is a crystalline substance, soluble in water, with a faint sweetish taste. With pure yeast it does not ferment. By the action of certain other fermenters, however,

it undergoes alcoholic fermentation, with the production at the same time of lactic acid, forming the drinks known as "koumiss," when made from mares' milk, and "kephyr" when from cows' milk. The ordinary souring of milk is due to the formation of lactic acid from the lactose by micro-organisms.

Milk sugar gives with many reagents the same results as glucose. It can be distinguished from glucose by its not fermenting with yeast and by its having a less strong power of reduction. It is unable to reduce cupric compounds to cuprous in acetic acid solutions (Barfoed's test).

63. **Preparation of Milk Sugar.**—Dilute 200 c.c. of milk with 800 c.c. of water, and add very cautiously not more than 0.1 per cent. of acetic acid to precipitate the casein (when enough has been added the liquid is nearly clear). Filter. Boil the filtrate and filter off the coagulated albumin. Evaporate the filtrate upon a water bath to a syrup and allow it to stand until the sugar has crystallized out. It may be purified by recrystallizing it.

64. Test milk sugar with Trommer's and the phenylhydrazine tests, and notice that the results are similar to those obtained with glucose.

65. Try Barfoed's and the fermentation tests as made with the glucose, and observe that the results are negative. (If over a 1 per cent. solution of lactose is used there may be some reduction of the cupric compound).

66. Boil a milk sugar solution with a little hydrochloric acid, neutralize, and try Barfoed's test. Glucose has been formed and this will now reduce the reagent.

SUCROSE (CANE SUGAR: $C_{12}H_{22}O_{11}$).

Cane sugar is found in plants, not in the animal kingdom. It has no reducing power and does not respond to

the tests where such a reducing action occurs, such as Trommer's, Fehling's, and Böttger's. It is decomposed by heating with acid into a molecule of glucose and one of fructose.

67. Apply two reduction tests to a solution of pure cane sugar. It gives no results.

68. What does this indicate about its molecular structure?

69. Boil a solution of cane sugar with a little sulphuric or hydrochloric acid, neutralize the solution, and prove that it contains glucose.

70. Equation for action of acid?

71. Resorcinol-HCl reaction for ketose sugars (Seliwanoff's reaction). The reagent is a 0.05 per cent. solution of resorcinol in 10 to 12 per cent. HCl. To 5 c.c. of the reagent in a test tube add a little of the invert sugar solution and boil, not more than 20 to 30 seconds; then let it cool. A red color appears and a brown precipitate. The latter is soluble in alcohol to a red solution.

What is the ketose sugar here? Why so called?

72. By means of the tests used, how can a distinction be made between dextrose, lactose and sucrose?

73. The "invert sugar" which results from the decomposition of cane sugar by acids can be separated into glucose and fructose by adding to 10 parts 6 parts of calcium hydroxide (slaked lime) and 50 parts of water. Both sugars form calcium compounds. That with glucose, being liquid, can be pressed out of the fruit sugar compound, which can then be decomposed, the fruit sugar being set free, by the addition of oxalic acid as long as a precipitate is produced. Filter and obtain the fructose by the evaporation of the filtrate.

MALTOSE (MALT SUGAR: $C_{12}H_{22}O_{11}$, H_2O).

74. Boil a small lump of starch with 25 c.c. of water, cool it to nearly body temperature, and add an aqueous

extract of ground malt, made at the latter temperature. Use 1 to 2 grams of ground malt to 10 to 15 c.c. of water. A high temperature will destroy the malt. Observe that the mixture becomes thinner, and that, finally, a sample is not turned blue by iodine. Then test for the maltose with phenylhydrazine, as in the glucose reactions. Try also Trommer's test or Fehling's test. It responds to all.

75. Preparation of Pure Maltose.—One hundred grams of starch are to be mixed with 500 c.c. of cold water as thoroughly as possible, then heated on a water bath until it makes a paste. Make a watery extract of malt at 40°C. from 6 or 7 grams of dry malt. When the starch-paste has cooled down to 60° or 70°, add the malt-extract and keep it at this temperature for an hour. When the starch has been converted to maltose the liquid becomes thin and watery. Then boil, filter, and evaporate to a syrup upon the water bath. Dissolve the maltose from the residue with small portions of 90 per cent. alcohol. Distill the alcohol off from this solution, and evaporate to a syrup. Let this stand until it crystallizes. This may be hastened by the addition of a little crystallized maltose, which can be prepared by evaporating a few drops of the solution in a thin layer on a piece of glass. It may be purified by recrystallizing from methyl alcohol.

PENTOSES.

Normally but small amounts of the pentoses are found free in the body, and these doubtless originate mostly in certain vegetable foods. In pathological conditions they may be more abundant. Like glucose, they are reducing agents, responding to Trommer's and Fehling's tests. They also form osazones which, however, have different melting points from the glucosazone. They do not ferment with yeast, nevertheless they may be mistaken for reducing sugars because of their other reactions. Arabinose, one of the most common, can be obtained by boiling cherry-gum with acid.

76. Prepare arabinose by heating a small piece of cherry-gum or gum arabic for 10 minutes with 5 c.c. of concentrated HCl. Neutralize and use for tests.

REACTIONS OF THE CARBOHYDRATES

	Iodine	Reduction of Fehling's Reagent	Phenyl-hydrazone	Solubility in Water	Fermentation with Yeast	Solubility in Alcohol	Taste	Crystallize	Polarized Light
Starch	Blue	—	—	Colloid on Heating	—	—	—	—	Right
Dextrins	Red to Yellow	*	+	+	—	—	Sweetish	—	Right
Glycogen	Red to Violet	—	—	Opalescent	—	—	—	—	Right
Cellulose	—	—	—	—	—	—	—	—	...
Saccharose	—	—	—	+	Only after Inversion	Slight	Sweet	+	Right
Maltose	—	+	+	+	+	Slight	Sweet	+	Right
Lactose	—	+	+	+	—	—	Sweet	+	Right
Glucose	—	+	+	+	+	Slight	Sweet	+	Right
Fructose	—	+	+	+	+	+	Sweet	+	Left
Arabinose	—	+	+	+	—	—	Sweet	+	Right

* Commercial (impure) dextrin does reduce.

Compare the reducing power of the original gum and the pentose formed from it.

As a substitute method a pentose can be prepared from straw.

77. Preparation of Xylose from Wheat Straw.—Use 100 to 500 grams of the chopped straw, depending upon the amount of the pentose desired. Let it stand twenty-four hours with an excess of 2 per cent. ammonia, then wash it with water to remove albumin, salts, etc. Boil the residue for about six hours with ten times the original weight of 2 per cent. sulphuric acid, keeping up the volume as it evaporates by adding water. Press out the liquid from the straw and filter, then neutralize the filtrate with commercial calcium carbonate (prepared chalk). Cool, filter out the calcium sulphate, and concentrate the filtrate on the water bath to a syrup. Extract the syrup with alcohol, filter, and allow the alcoholic filtrate to evaporate, when the

xylose should separate in the crystalline form. The yield should be about 5 per cent.

78. Saturate 5 c.c. of hydrochloric acid (30 per cent.) with phloroglucin (about 25 mg.). Add to this the solution which is to be tested for pentoses and heat just to boiling. The presence of pentoses is indicated by a red color, and this shows absorption bands between D and E of the spectrum. Glycuronic acid gives a similar result.

79. Repeat the test as in the last experiment, substituting orcin for phloroglucin. Pentoses produce a green color. Glycuronic acid does not act in this manner unless a considerable amount is present or the boiling is unduly protracted.

80. How can a pentose be separated from dextrose?

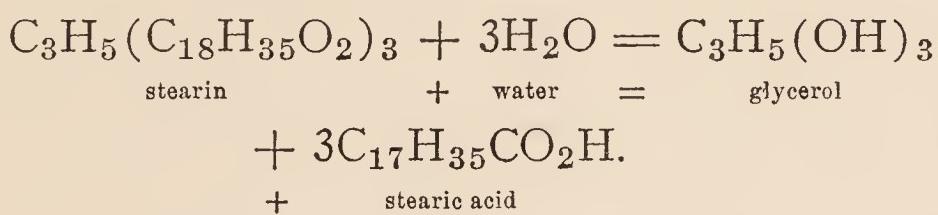
THE LIPOIDS.

Lipoids are organic animal or vegetable compounds which are soluble in ether and similar solvents.

THE FATS

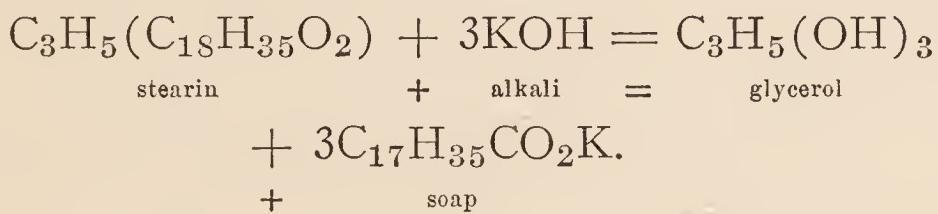
The fats occur in both plants and animals. When pure they are colorless, odorless, and tasteless. They are insoluble in water and have a lower specific gravity. They dissolve in hot alcohol more easily than in cold, and are easily soluble in ether, gasoline, or benzene. The fats mix with water when the two are shaken violently together, but they soon separate, the fats going to the top. If, however, something like soap or a solution of albumin is added to the mixture, which will form a coating around the minute globules of fat, they are prevented from reuniting, and form an emulsion,—that is, a mixture of small fat globules with the liquid, not a solution. It is destroyed by anything which will remove the coating, the fat separating again from the liquid. The fats are com-

posed of three elements: carbon, hydrogen, and oxygen. They contain a much smaller percentage of oxygen than the carbohydrates, the hydrogen and oxygen not being in the proportion to form water. When the fats are kept at the temperature of superheated steam or subjected to the action of the pancreatic ferment, or other catalytic agent, they take up water and are split into two compounds: glycerol, on the one hand, and one or more of the fatty acids, on the other. Thus, stearin gives



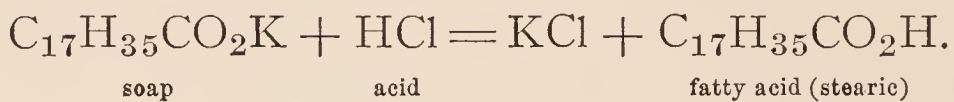
They may be considered, then, as made up of glycerol and a fatty acid less water.

This splitting up of the fat molecule is called saponification. It occurs when fats become rancid. It can be also effected by boiling the fat with a caustic alkali. Here, instead of the free fatty acid being left, it unites with the alkali to form a salt. These metallic salts of a fatty acid are the soaps:—



The soaps of the alkalies are soluble in water, the potassium compound being hygroscopic and forming soft soap. The sodium compound forms a hard soap. The compounds of the heavy metals with the fatty acids are insoluble, and can be formed by adding a solution of their salts to a soap solution. The lead soap or lead plaster

used in medicine is made by heating lead oxide with one of the fats. The soluble soaps can be thrown down from their solutions by saturation with a neutral salt. If a strong acid is added to a soap solution the soap is decomposed, the metal uniting with the strong acid and the fatty acid being set free as an insoluble substance:—



These fatty acids which enter into the most of the animal and vegetable fats are one of the unsaturated series: oleic acid ($\text{C}_{17}\text{H}_{33}\text{CO}_2\text{H}$); and two of the saturated series: stearic acid ($\text{C}_{17}\text{H}_{35}\text{CO}_2\text{H}$) and palmitic acid ($\text{C}_{15}\text{H}_{31}\text{CO}_2\text{H}$). Besides these acids—which constitute, by far the larger part of those present in fats—there are found in some cases certain of the lower members of the saturated series, such as butyric ($\text{C}_3\text{H}_7\text{C}-\text{O}_2\text{H}$), caproic ($\text{C}_5\text{H}_{11}\text{CO}_2\text{H}$), caprylic ($\text{C}_7\text{H}_{15}\text{CO}_2\text{H}$), and capric ($\text{C}_9\text{H}_{19}\text{CO}_2\text{H}$), which occur in butter.

The acids which enter into the fats resemble the latter in many of their properties. They differ from them in having a slight acid reaction, the fats being neutral. They can also be distinguished by their not giving the irritating odor of acrolein, as the fats do when they are heated. This is produced by the decomposition of glycerol, either in a fat or when heated alone. It is most easily obtained by adding before heating some substance which will assist in removing the water. The chemical change is



The free acids can be neutralized by even weak alkalies, forming the soaps.

The animal and many of the vegetable oils are true fats, differing only in that they are liquids at ordinary temperatures instead of solids. We must, however, distinguish between these and the essential or volatile oils which are found in plants, but which are not fats. The fats produce spots on paper which are not volatile and do not disappear on standing. The essential oils will disappear when left exposed to the air. The mineral oils belong to an entirely different class of compounds, and do not contain oxygen.

The fats are named from the acid which they contain. Thus the compound of stearic acid is called stearin; of palmitic acid, palmitin; and of oleic acid, olein. Sometimes the prefix *tri* is used with these names, as tristearin, etc. They differ principally in their melting points, olein being a liquid at ordinary temperatures; palmitin and stearin, solids, the former melting more easily than the latter. In the animal body these fats are usually mixed, the consistence of the fat varying with the composition. Thus, the fat of the ox, or tallow, is a firmer solid than the fat of the hog, or lard, because it contains less of the olein. The animal oils contain more olein than stearin and palmitin, consequently they melt at temperatures below the ordinary ones, and are liquids. Human fat contains 67 per cent. to 80 per cent. of olein.

81. Show that cottonseed oil is not a simple fat, but a mixture, by cooling it to the freezing point either by natural cold or by a freezing mixture. It separates into two parts: one crystalline, consisting mostly of palmitin, and the other olein, which remains liquid.

82. Is this a chemical or a physical change? Reasons?

83. Try the reaction of a fresh fat, like lard, cotton-seed, or olive oil, with a piece of litmus paper. It is neutral; but, if the fat has been standing some time and has become rancid, it may be slightly acid.

84. Try the solubility of a few drops of cottonseed oil in a test tube of water. It mixes when shaken violently, but soon separates at the top on standing. Add now a few drops of a soap solution and shake again. The liquid becomes milky and the fat does not separate. If the oil is not fresh it may be necessary to add a few drops of sodium carbonate to neutralize the free acid.

85. Examine a drop of the emulsion so formed under the microscope. It will be found to consist of a great number of minute globules, the size being smaller the more thoroughly the liquid is shaken. They are kept apart by the thin film of soap which covers each one.

86. Add a small amount of hydrochloric acid and shake. The soap will be decomposed and the fat will collect at the top, as at first.

87. Try the solubility of a fat in ether, chloroform, or gasoline, avoiding carefully the vicinity of a flame. It is easily soluble.

88. Is it changed chemically. Proof?

89. Try the solubility of a piece of tallow or lard, the size of a pea, in twice its volume of warm benzine. It is readily soluble, and separates on slowly cooling in the crystalline form. The crystals can be examined with the microscope. Sketch these.

90. Show that the fats are non-volatile by placing a little upon paper and warming it over a flame. It does not disappear.

91. In a dry test tube heat to a high temperature a piece of lard as large as a pea and note the odor of acrolein which irritates the eyes and membranes of the lungs and throat.

92. Is there a chemical change in the fats of foods when they are cooked by roasting? by frying? by boiling? If there is a chemical change in either case, would it be likely to make the fat more edible?

93. To about 10 grams (11 c.c.) of cottonseed oil add 20 c.c. of 10 per cent. alcoholic solution of potassium hydroxide. Heat the mixture gently in a flask, stirring meanwhile, until the odor of the oil has largely disappeared and it appears homogeneous and no oil separates when a few drops are poured into water. The product is a mixture of potassium soap and glycerol.

94. Why is the change more rapid with an alcoholic than with an aqueous solution? Would the solvent action of the hot alcohol on the fat account for it? Why heat gently instead of strongly?

What is the name of this type of chemical reaction? What is the chemical name for the fats as a class of compounds? Classify glycerol as a chemical compound.

95. Convert a portion of the soap into the sodium, or hard soap, by adding some saturated salt solution and allowing it to stand until cold. Will it dissolve on warming?

96. Dissolve another portion in water and add a calcium solution. A calcium soap is formed which is insoluble in water. It is this compound which is produced by the action of soap on "hard water." Many of the heavy metals give similar compounds. Try it with solutions of iron, lead, copper, etc.

97. What are the chemical names of these soaps of Ca, Fe, Pb, and Cu?

98. Dissolve the remainder of the potassium soap solution in a little hot water and add dilute sulphuric acid slowly until it is plainly acid to test paper. The fatty acids are set free as insoluble substances, the glycerol remaining in solution. Filter out the acids by means of a wet filter paper, through which they will not pass. Save the filtrate for the extraction of the glycerol in 106. Wash out the sulphuric acid with distilled water until the wash water is no longer acid, and try the reaction of the fatty acids with litmus paper. They are acid to litmus.

99. How does the degree of acidity compare with that of dilute sulphuric, hydrochloric or acetic acid? Why?

What kind of soap would be made if the lye obtained by the leaching of wood ashes were heated with fats? Why?

100. Dissolve about 0.1 gram each of stearic acid (a saturated acid) and oleic acid (an unsaturated acid) in 2 to 3 c.c. of chloroform. Add to each a few drops of Hübl's iodine solution (26 grams of iodine and 30 grams of mercuric chloride dissolved in 1 liter of alcohol). Note that the unsaturated acid decolorizes the iodine solution and the saturated acid does not.

101. In the above manner test the following fats and oils to learn if they contain unsaturated acids—cottonseed oil, lard, olive oil, lubricating oil, or any edible fats.

102. What is molecular difference between a saturated and an unsaturated organic compound? Show this by the formula of oleic acid, $C_{17}H_{33}CO_2H$, comparing it with that of stearic acid, $C_{17}H_{35}CO_2H$. What becomes of the iodine when it is decolorized? Equation?

103. Dissolve the fatty acids in hot alcohol, let this cool slowly, observe and sketch the crystals.

104. Allow the fatty acids to stand until the water has drained off or dry them by the aid of filter paper. Heat them in a dry

test tube until they commence to decompose, and see if they give the irritating odor of acrolein. If the acids were washed clean, and if the fat was completely saponified, this should not appear. Try the same test on any of the fats, and the odor will be given.

105. Why is acrolein formed from fats and not from fatty acids?

106. Neutralize the first filtrate from the acids, which contains the glycerol, by adding a little powdered chalk, then evaporate the mixture to complete dryness on a water bath. Extract the glycerol from the powdered mass by alcohol and evaporate this on the water bath. If the glycerol is to be further purified this residue should be extracted with absolute alcohol and the alcoholic filtrate evaporated. The thick liquid is the glycerol, as is shown by the taste. Glycerol dissolves many metallic oxides, *e.g.*, precipitated, washed cupric hydroxide dissolves to a blue liquid. Where have you seen this?

107. Remembering the composition of the fat and what has been added to it, give a list of all compounds contained in the dried residue before treating with alcohol.

108. Mix 2 grams of lead monoxide (litharge) with 11 c.c. of olive oil and 75 c.c. of water. Boil until the oil is entirely converted into the lead soap. This is "lead plaster." Filter it off; wash the insoluble soap with water and evaporate the filtrate with the wash water in an evaporating dish on a water bath, as long as its volume decreases. The glycerol remains. It contains lead; if the pure substance is desired this can be removed by diluting with water and passing hydrogen sulphide into it.

109. **The Preparation of Pure Palmitic Acid.**—Melt in hot water 10 grams of bayberry wax, which is nearly pure palmitin, and add 20 c.c. of a 20 per cent. solution of sodium hydroxide. Boil it until a drop makes a soapy solution when poured into a test tube of water. Sodium palmitate has been formed together with glycerol, carbon dioxide and water. (Write the equation for the reaction.) Acidify slightly the soap solu-

tion with dilute hydrochloric acid, filter out the palmitic acid, wash it with cold alcohol until it is white, dissolve it in the least possible quantity of hot alcohol, and let it stand until it is cold. Examine with a microscope the crystals which have separated and make sketches of them. Test the reaction to blue litmus paper.

110. The Preparation of Pure Soap.—Melt at a low temperature most of the palmitic acids from the last experiment and slowly add sodium hydroxide solution until the reaction is slightly alkaline, warming meanwhile. A hard soap is produced. (Write the equation.) If a little alcohol is mixed with this before it cools a transparent soap results. Why?

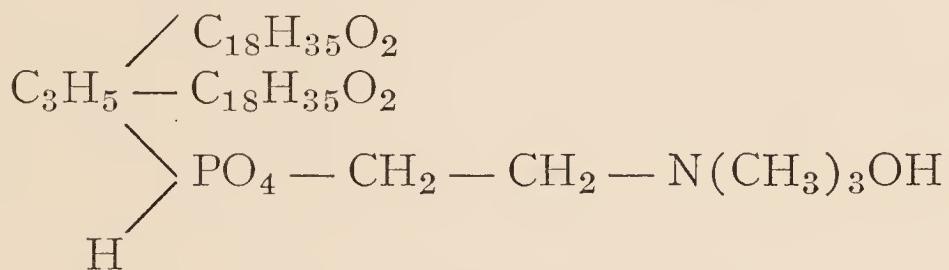
111. Hold a piece of small glass tubing in the Bunsen flame until it is soft, turning it continually. Then draw it out into a capillary tube about six inches long. Break this in the middle, forming two open capillaries each with a large end. Melt a fat at low temperature and draw it up into the capillary by exhausting the air, allowing it to cool and solidify there. Seal up the large opening by means of a blast lamp or Bunsen burner. Attach the tube to the bulb of a thermometer by means of a rubber band cut from a piece of tubing. Hold this in a beaker of water, warming the latter slowly. Make a note of the melting point—the temperature at which the fat liquefies. In this manner determine the melting points of a number of fats, like mutton tallow, beef tallow, lard, butter, also of palmitic acid and stearic acid. Hand in the results. Place the thermometer with the capillary of melted fat in water a little above its melting point and let it cool slowly, noting the point at which solidification occurs. Notice that this is below the melting temperature and that it varies with the speed of cooling.

112. Try the solubility of fats, fatty acids and soaps in chloroform, ether, water, hot and cold alcohol. Tabulate the results and determine what methods can be employed for their separation.

113. Make the acrolein test upon the water-free glycerol by heating in a dry tube. The glycerol is decomposed, giving the acrolein odor, and showing that the odor when obtained from fats is due to the glycerol radical, and not to the acid part.

THE LECITHINS.

The lecithins are found in nearly all animal and vegetable cells. They are very abundant in the brain and nerves and in the yolk of eggs. They are sometimes called phosphorized fats. The formula of one of those most common in the animal body is $C_{44}H_{90}NPO_9$, and the composition is probably



It is therefore a fat where for one stearic acid radical has been substituted the group



In others, instead of the stearic acid radical we may have the oleic or palmitic radical. Like other fats, they can be decomposed or saponified. They then give phosphoric acid, a fatty acid, and a base, like cholin:—



Lecithin is a soft, waxy substance, which swells in water to a pasty mass. This, under the microscope, has the form of oily drops or threads, the so-called "myelin" forms. It resembles nuclein in the readiness with which it unites with albuminous substances. It is found in the yolk of the egg in an unstable union with vitellin.

114. Preparation of Lecithin.—In the following work the student should remember that ether and petroleum-ether are very inflammable.

Separate the albumin of an egg as completely as possible from the yolk. Place the yolk in a cylindrical, glass-stoppered bottle, add two or three times its volume of ether, and shake the bottle until they are well mixed. Allow it to stand until the ether above becomes clear, then decant the latter into a distilling flask. Repeat this extraction several times, when most of the coloring matter should have been dissolved. Preserve the insoluble for the preparation of hematogen. Mix the portions of ether and distill off the ether. The residue contains the lecithin mixed with fats, cholesterol, and coloring matters. Dissolve this in petroleum-ether and filter. Pour the filtrate into a separatory funnel, add about one-fourth its volume of 75 per cent. alcohol, and shake. When the two liquids have separated, draw off the alcohol, which contains most of the lecithin. Repeat this extraction with alcohol several times and unite the alcoholic solutions. Distill off the remainder of the petroleum-ether from these, and let the solution stand several days in a cool place. A precipitate of cholesterol and other impurities will form, from which the solution is to be decanted through a filter. Boil the filtrate with a little animal charcoal to decolorize it, and filter. Evaporate at a temperature of 50° to 60° to a syrupy consistency. Cool this and dissolve in ether. If it does not dissolve completely, filter it. Evaporate the ether, when the lecithin remains nearly pure. If desired, it can be purified further by dissolving in as small an amount as possible of warm absolute alcohol and placing this in a freezing mixture of —5° to —15°, when the lecithin crystallizes out. It should be filtered in the cold.

115. Place a little lecithin in water and examine with the microscope. Notice the myelin forms.

116. Warm this mixture with water and notice that after a time the lecithin turns brown and the reaction becomes acid from decomposition.

117. Boil a portion with sodium hydroxide. Notice the fishy odor of trimethylamine from the cholin.

118. Mix a little lecithin with dry, powdered potassium nitrate in a small porcelain crucible, and warm, at first gently, then, after deflagration, until the dark color has disappeared. After cooling

dissolve in water and test for phosphoric acid by nitric acid and ammonium molybdate. At once or after warming a yellow precipitate will appear.

119. Use a small portion for the acrolein test (91).
120. What do each of the above tests show about the molecular composition of lecithin?

THE PROTEINS.

The protein compounds constitute the greater part of the solid matter of the blood, muscles, nerves, and other organs of the animal body. The urine, tears, and perspiration, in a normal condition, never contain more than a trace. The proteins contain carbon, hydrogen, nitrogen, oxygen, and usually sulphur. Some contain phosphorus and a few others iron. When heated they are charred, giving off water, inflammable gases, and ammonia, at the same time emitting a strong odor, similar to that of burnt horn or wool. Upon further ignition they leave an ash, though whether this was originally a part of the protein molecule has not been decided. They are often spoken of simply as the nitrogenous constituents of the body or the food, although not all of the nitrogenous compounds found there belong to this class.

The proteins are very complex substances with a high molecular weight, and it is probably owing to this fact that they are so easily decomposed, as is seen by the putrefaction which sets in soon after life has ceased. To the large molecule, too, is due the inability of most of them to pass through a parchment or animal membrane.

GENERAL PROPERTIES OF THE PROTEINS.

121. Burn a small piece of dry albumin or other protein compound on a piece of porcelain or platinum foil,

or on a wire. Notice that it turns black from the presence of carbon. Observe the characteristic odor. On continued heating it will all disappear except the mineral matters, or ash.

122. What is the relative amount of ash in protein?

123. Mix a few fragments of dry albumin with an excess of powdered soda-lime and heat the mixture in a dry test tube. Test the vapors which escape for ammonia, both by the odor and by their action, on a piece of red litmus paper. What elements are thus proved present in the protein molecule?

124. Would this experiment show that nitrogen is abundant or slight in amount?

125. **Test for Sulphur in Protein by Oxidation.**—In a porcelain crucible or on a crucible cover fuse a little dry protein with twice its bulk of an alkaline oxidation mixture, composed of equal parts of dry sodium carbonate and sodium nitrate. Continue heating until the residue is nearly colorless, then cool, treat with water, filtering if it does not dissolve to a clear solution. Acidify the filtrate with HCl and add a barium solution. A fine white precipitate of BaSO₄ proves the presence of sulphur.

126. Explain the chemical change during the fusion, with function of each constituent of the reagent. Equation for precipitation? What forms of sulphur can be thus precipitated by a barium solution and what ones cannot?

127. Make an alkaline lead solution by adding sodium hydroxide to a small amount of lead acetate solution until the precipitate first formed has dissolved. Add to it a protein compound, like albumin, and boil. The presence of sulphur (cystine sulphur, or unoxidized sulphur) in the protein compound is shown by the dark colored lead sulphide, which it forms by uniting with the lead.

128. What is cystine?

129. Heat a little bismuth subnitrate in a solution of albumin which has been made strongly alkaline with sodium hydroxide, and notice that the sulphide of bismuth, which is formed, has the same appearance as the metal which is produced by the glucose with the same reagent (51); that is, albumin gives a similar result to that obtained with dextrose.

130. Which properties shown so far would best distinguish albumin from dextrose?

131. **Test for Sulphur in Proteins by Reduction.**—Very small quantities of sulphur may be shown by changing it to a sulphide and testing for the latter with sodium nitroprusside. By this means it can be found in a minute piece of dry albumin. Close the air vent of a Bunsen burner and, after turning it low, cover the albumin with moistened sodium carbonate and hold it on a platinum wire, or an iron one from the wire gauze, in the middle of the flame. Allow the substance to fuse, being careful to keep it in the yellow flame to prevent oxidation. Then dissolve the mass in a drop of water in a porcelain dish. Add to the solution a very small crystal of sodium nitroprusside or a drop of a dilute solution. The presence of sulphide is shown by the production of a purple or violet color, which is destroyed by an excess of the nitroprusside. Explain all chemical changes.

132. Test a solution of egg albumin or any other albuminous substance to see if it will pass through a dialyzer. Only the proteoses and peptones will pass through the membrane. The biuret test can be used to detect them (133).

REACTIONS OF PROTEINS WHICH CAN BE USED FOR IDENTIFICATION OF THE GROUP.

Color Reactions.—Many reagents give colored products with the proteins. The recent advances in our knowledge of the components of the protein molecule, as shown by the isolation of the products of its hydrolytic decomposition, enable us to say which of these components are the cause of some of the individual colors.

133. Biuret Reaction.—Make 3 to 5 c.c. of an egg albumin solution alkaline with as much sodium hydroxide solution, then slowly drop in not more than four or five drops of a very dilute copper sulphate solution. A color appears, violet to pink, according to the protein used.

This color is produced by compounds containing two amino complexes united by nitrogen or carbon, for example, two CONH₂ groups or one CONH₂ with one CSNH₂. The compound called biuret,



gives the color, although it is not a protein.

134. Xanthoproteic Reaction.—To 2 to 3 c.c. of an egg albumin solution in a test tube add 1 c.c. of concentrated nitric acid and heat to boiling; the liquid or coagulated mass turns yellow. Make it alkaline; the color becomes a deeper yellow or orange.

The action of the nitric acid on derivatives of the phenyl group, C₆H₅—, is the cause of the yellow color.

135. Millon's Reaction.—Millon's reagent is a solution of the nitrates of mercury in nitric and nitrous acid. It is prepared by dissolving one part by weight of mercury with two parts by weight of concentrated nitric acid (sp. gr. 1.42) and diluting the solution with two volumes of water. If a sediment remains decant the reagent off from it. It is most satisfactory with solid proteins or those which coagulate on heating in an acid solution.

To 3 to 4 c.c. of egg albumin solution add a few drops of Millon's reagent. A white precipitate appears, which turns red upon heating.

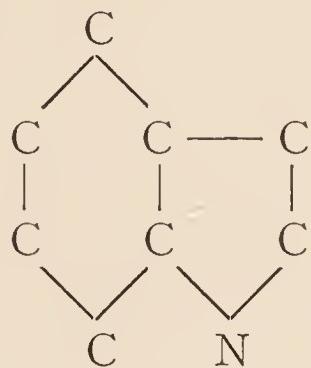
The reaction is due to the presence of the hydroxyphenyl radical, —C₆H₄OH, since other organic com-

pounds which contain it give the color. It is interfered with by chlorides or other substances which precipitate mercury.

136. Glyoxylic Acid Reaction (Hopkins-Cole reaction).—The reagent is prepared, by Benedict's method, by placing 10 grams of powdered magnesium in a liter Erlenmeyer flask, mixing it with 100 to 200 c.c. of water, and slowly pouring in 250 c.c. of a saturated solution of oxalic acid. Much heat is liberated, and the flask must be kept cold by running water. Filter out the insoluble magnesium oxalate, wash the filter slightly, acidify the filtrate with acetic acid, and dilute with distilled water to one liter.

Mix 2 to 3 c.c. of an egg albumin solution with as much Hopkins-Cole reagent in a test tube, slant the tube and let about 5 c.c. of concentrated sulphuric acid run under the mixture. A purple ring forms at the junction of the liquids. If the liquids are mixed the color spreads through the whole.

The indol group,



or its derivatives, like tryptophane, is the cause of the color change. Proteins like gelatin, in which it is absent, do not give the reaction.

Many proteins can be precipitated from their solutions by means of some salts without any apparent chemical

change in their composition or properties. Sulphates and chlorides of ammonium and the light metals will precipitate them in this manner. When such salts are removed, or the precipitated proteins are treated with water, they will dissolve.

More active reagents, like strong acids or alkalies, salts of the heavy metals, or alcohol after long contact, do chemically change (denature) the proteins. The fact that a protein redissolves after precipitation is not proof that there has been no chemical change, inasmuch as their composition or other properties may have been altered.

PRECIPITATION REACTIONS.

137. To 3 to 5 c.c. of a rather concentrated solution of egg albumin in a test tube add a drop or two of acetic acid and stir in sodium chloride crystals until a precipitate forms. Filter or decant the liquid and try the solubility of the precipitate in water. Try the biuret test, and any others you choose, and decide whether there was a chemical change with the first precipitation.

138. To 3 to 5 c.c. of a rather concentrated solution of egg albumin add 95 per cent. alcohol until a precipitate forms. Let half stand over night in the alcohol. Test the other half as in the preceding experiment, and the next period test the second half in the same manner. Is the albumin chemically changed?

139. Slightly acidify an egg albumin solution with acetic acid and boil. Is the coagulation a chemical change?

140. Precipitate a rather concentrated solution of albumin with solutions of CuSO_4 and with HgCl_2 . Drop in the reagent slowly. Does an excess alter the result? Is there a chemical change?

141. Use picric acid as the precipitating agent and answer the same questions.

142. Make the solution of albumin acid with acetic acid, then add at least an equal volume of a saturated solution of ammonium sulphate, and heat to boiling. Most simple proteins are thrown down as a white precipitate. Are they chemically changed?

143. Acidify the solution of albumin with acetic acid and add a few drops of potassium ferrocyanide. A white precipitate is formed.

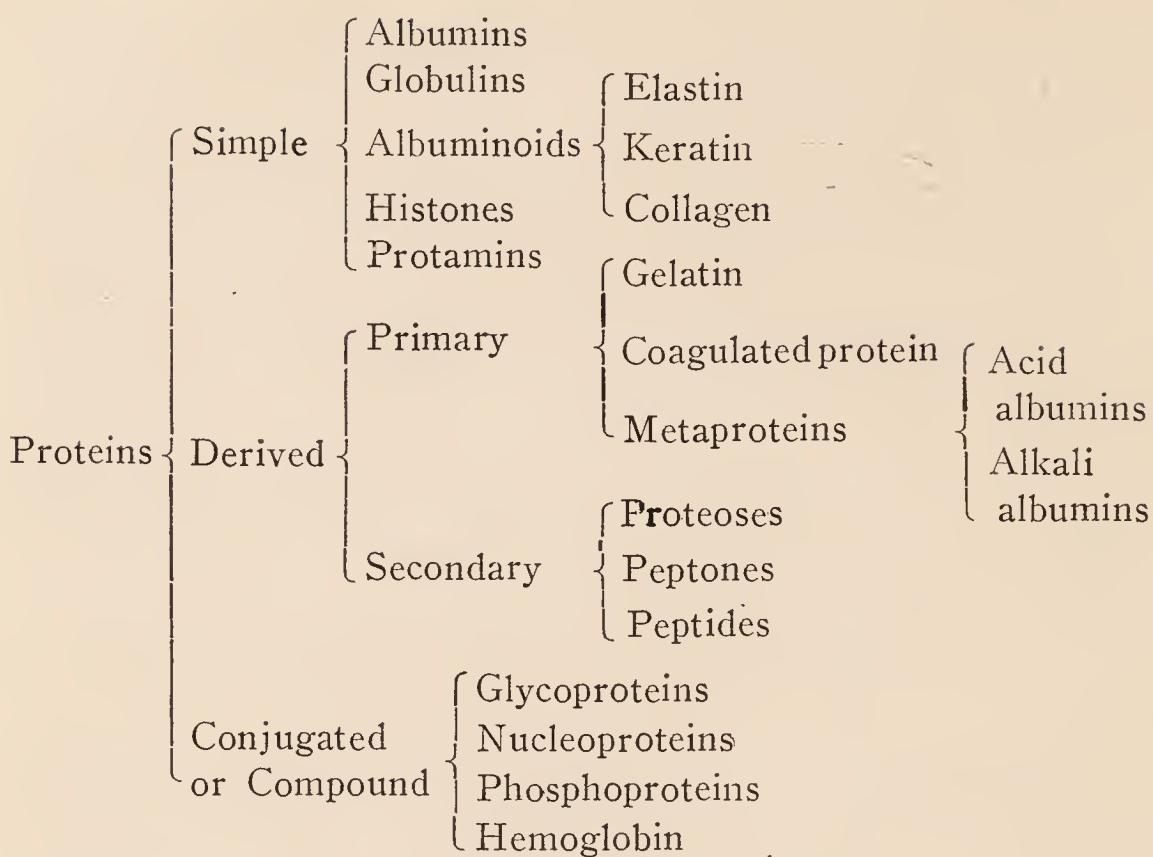
The two last reagents fail in case of the peptones.

144. Try also ease of precipitation of an albumin solution by (1) trichloracetic acid, (2) metaphosphoric acid, (3) phosphotungstic acid from a solution acidified by a mineral acid, (4) tannic acid from a solution acidified by acetic acid.

145. Which of the above protein reactions could be used to separate one protein from another? Which would best detect small amounts without separating them?

CLASSIFICATION OF THE MORE COMMON PROTEINS.

The protein group of compounds can be divided into a number of classes. The simple proteins are those which yield by hydrolytic decomposition only alpha amino acids. The derived proteins are products of such a hydrolytic decomposition, intermediate between the native protein and the amino acid. The primary ones are first produced and are only slightly altered in composition and properties; the secondary ones have the protein molecule decomposed to a farther stage. The conjugated proteins are compounds of a simple protein with some non-protein organic compound.



THE SIMPLE PROTEINS.

These form the principal part of the protoplasm which is found in animal and plant cells. Upon hydrolysis they yield alpha amino acids or their derivatives, but the constitution of the molecule and even the exact formula of some of the simplest members of the group is uncertain. They are known to be very complex, those which have been most studied having several hundred atoms in a molecule. They differ somewhat from each other in composition, but their constituents usually lie within the following limits:—

Substance.	Average of Most Analyses.	Approximation.
C	50.0 to 55.0 per cent.	52 per cent.
H	6.5 to 7.3 per cent.	7 per cent.
O	20.0 to 23.5 per cent.	23 per cent.
S	0.3 to 2.2 per cent.	2 per cent.
N	15.0 to 18.0 per cent.	16 per cent.

Phosphorus is sometimes found in less amounts than one per cent.

A few of the simple proteins have been obtained in a crystallized form but most of them are amorphous. They differ in their solubilities and are classified largely upon this basis. The peptones will diffuse through an animal membrane, but they do not pass through rapidly.

The simple proteins, like some other organic compounds which do not belong to this class, are thrown out of solution when to the solution certain neutral salts are added until it is saturated. Ammonium sulphate will precipitate all but the peptones and perhaps a few of the proteoses. Magnesium sulphate and sodium chloride will precipitate many of them.

When the simple proteins are heated with water, many of them are coagulated, passing into an insoluble modification. The temperature at which this takes place is called the coagulation point. This is a different one for most of the different substances, and may be used in their identification and separation. It may vary, however, from the presence of other substances. It may be raised, prevented, or the coagulation made incomplete by alkalies or by some organic acids, like acetic acid. Coagulation is favored and the coagulation point is lowered in the presence of neutral salts or small amounts of a mineral acid. The concentration of the solution also may make it vary. Through coagulation the nature of proteins is altered and they acquire other properties. By the action of alcohol they are precipitated, at first in an unaltered form; but if the alcohol is strong and acts for some time they are coagulated, and are then insoluble in water.

Coagulation, when spoken of with respect to the protein compounds, must be distinguished from precipita-

tion, which it resembles. When albumin is coagulated—*e.g.*, by boiling, by mineral acids, or by the continued action of strong alcohol—it becomes insoluble in water. It may be precipitated by ammonium sulphate without being coagulated or by not too large an amount of alcohol and still retain its original properties, being soluble again upon the addition of water.

Some of the simple proteins are coagulated by the action of ferments; for example, the fibrin, which is so formed from the blood or lymph.

Simple proteins are easily decomposed by the action of the putrefactive bacteria, the nitrogen and sulphur uniting with hydrogen to form hydrogen sulphide, and ammonia, or, these two together, ammonium sulphide. Other nitrogen compounds are also formed, like the amino acids which contain the amino group, NH_2 , such as leucine and tyrosine. Indol is also one of the nitrogenous putrefactive products.

Many of the simple proteins are precipitated by the mineral acids, but upon standing with an excess of the acid, or more quickly by heating, they are dissolved, going into acid albumins. Many will also form insoluble compounds with salts of the heavy metals, such as mercury, copper, and lead. With copper in an alkaline solution they give a blue or purple color and upon boiling with an excess of nitric acid a yellow, which becomes more reddish upon being rendered alkaline. Millon's reagent, which gives a red with all compounds containing a benzene nucleus united with an hydroxyl group, produces the same color with simple proteins, whence it is believed that the above complex is contained in these compounds. The xanthoproteic reaction is attributed to the same group.

146. Preparation of cystine ($\text{H}_2\text{CS}-\text{CHNH}_2\text{CO}_2\text{H}$)₂ from hair or wool (Folin's method).—Place in a flask about 50 grams of wool or hair (which can be obtained at the barber shop) in a 500 c.c. Erlenmeyer flask and add 100 c.c. of concentrated hydrochloric acid. Insert a stopper with a straight 7-10 mm. tube about three feet long, to prevent the escape of too much of the acid, and after solution has taken place boil it gently with a small flame for three to four hours. Add solid sodium acetate until Congo red paper shows no free mineral acid. (If you do not know the reaction, try Congo red with dilute hydrochloric or sulphuric acid). One hundred to 150 grams will be necessary. Let the mixture stand at least three days; three weeks will give a better yield. Filter out the crude cystine on a Buchner funnel.

Preserve the filtrate to obtain tyrosine.

Dissolve the crude cystine in 150 c.c. of 2 to 3 per cent. hydrochloric acid and boil for five to ten minutes with about 20 grams of purified bone-black to remove the color. To purify the bone-black let it stand over night covered with dilute hydrochloric acid, filter and wash until the filtrate is neutral. What is removed, and why would it be objectionable in this experiment? Filter again on a Buchner funnel. The filtrate must be perfectly colorless or the treatment with bone-black must be repeated.

Heat the filtrate to boiling and remove the hydrogen ion as before by sodium acetate. In this case a hot concentrated solution should be added very slowly, testing portions with Congo red in order to avoid an excess. Pure, white cystine should crystallize out on standing.

Examine the cystine crystals under the microscope and sketch them. Test them for sulphur. Is the sulphur the unoxidized form? (127.)

Let the precipitated cystine preparation stand until the liquid is perfectly cold, then filter out the cystine and use the filtrate to obtain the tyrosine. Concentrate it to a small bulk and let the tyrosine crystallize out. Examine with the microscope. (Plate II, 12c.) Preserve for testing later.

Albumins.

The albumins are simple proteins, soluble in water and coagulable by heat. They are not precipitated by dilute acids or alkalies. They are precipitated by saturation with ammonium sulphate and by some of the salts of the heavy metals, such as mercuric chloride and salts of copper, silver, and lead. For this reason albumin is successfully used as an antidote for poisoning by many of these metallic salts. By the action of acids it is converted into acid albumin and by the caustic alkalies into alkali albumin.

There are several different varieties of albumin usually named from their sources, such as the serum albumin of the blood; egg albumin; and the albumin of milk, or lactalbumin. We know that these are not the same substances from their physiological action, as well as from some chemical and physical properties. If, for example, egg albumin be injected into the circulation it usually passes in a short time into the urine. Serum albumin injected in the same manner does not pass unaltered through the kidneys. They differ also in their coagulation temperatures.

Albumin may be obtained for the experiments by dissolving the commercial dry egg albumin or serum albumin in cold water, filtering if it is not clear. From fresh eggs it can be prepared by separating the albumin from the yolk, being careful not to mix them. Then beat the albumin, to break up the membranes in it, mix with twice its volume of water, and filter through a piece of unbleached muslin.

147. Place a moistened piece of parchment or a parchment filter in a funnel from which the stem has been

broken, and hang the funnel in a beaker of water so that the water rises nearly to the top; or use a piece of parchment tubing, suspended in a beaker of water, with the two ends above the surface (Fig. 1). Into the parchment pour a solution of egg or serum albumin with a little salt, and let it stand all night. Test the outer liquid by the xanthoproteic and biuret reactions for proteins. None will be found, although it contains the chloride, as shown by adding silver nitrate with nitric acid, when a white curdy precipitate of silver chloride appears.

148. Close the stem of a thistle tube temporarily and fill the stem and bulb with an albumin solution; tie a moistened parchment over the bulb, invert and suspend the tube by a clamp, so that the bulb is covered by the water in a beaker below. The liquid slowly rises in the tube above the level of that outside owing to the inward osmotic pressure of the water being greater than the outward one of the albumin. This is the action that occurs when animal cells are surrounded by pure water.

149. **The Preparation of Crystallized Egg Albumin.**—To 100 c.c. of egg-white from fresh eggs add an equal volume of a saturated solution of ammonium sulphate. Beat the mixture thoroughly with an egg beater or a fork to break up the membranes and let it stand in a cool place for several hours, then filter through muslin or paper until the filtrate is clear. Add to the filtrate more saturated ammonium sulphate until a permanent precipitate forms, after which drop in distilled water until this dissolves, avoiding an excess. Now add acetic acid, drop by drop until a slight precipitate appears, and let it stand over night, when there should be a separation of the albumin in rosette like clusters of needle shaped crystals. If the eggs are not fresh a little more acid must be used—enough to make a fairly bulky precipitate—and then the solution must stand over night in a closed flask. To recrystallize, dissolve in cold water, add a few drops of acetic acid, then saturated ammonium sulphate solution

as before till the precipitate commences to form, and let it stand. The yield should be about 40 per cent.

150. Try the effect of various reagents upon the coagulation temperature of albumin by placing about 10 c.c. of an albumin solution in a number of test tubes, first filtering if necessary to obtain a clear liquid, and adding to each three drops of the following solutions (1) sodium chloride; (2) concentrated sodium chloride and acetic acid; (3) dilute hydrochloric acid; (4) sodium hydroxide; (5) a solution of sodium carbonate; (6) nothing. Set the test tubes in a beaker of water and heat to boiling, observing which coagulate first. It will be found that acids and neutral salts favor coagulation and that alkalies hinder it or prevent it altogether.

151. What is the order of coagulation of the tubes? Is it changed if the amounts of the added chemicals are varied?

152. In cooking foods which contain albuminous compounds are any of these modifying conditions ever present? Where?

153. To 3 to 4 c.c. of a rather concentrated solution of albumin add first a little, then more, then an excess of concentrated hydrochloric acid. Do the same with concentrated sulphuric acid. Under what conditions is the albumin here precipitated? Is it coagulated? Test by diluting with water.

154. What would be the action of acids of this class upon soft body tissues rich in protein-filled cells? Would they bring about any chemical change? How far could their action be overcome by washing off the acid?

155. Suspend a small beaker in a larger one, keeping them separate by wedges of cork; fasten by a clamp. Nearly fill both with water, and place in the inner one a test tube with enough solution of filtered egg albumin to stand at the same level as the water. The test tube should not touch the beaker wall. Heat the water gradually, having a thermometer in the albumin solution. Above 45° the temperature should not be allowed to rise

faster than 1° per minute. Make a note of the temperature where the liquid becomes opalescent and where a precipitate forms.

156. Put some of the clear albumin solution into three test tubes. Make one of them faintly acid by a drop of very dilute hydrochloric acid; add to the second enough of a very dilute solution of sodium carbonate to make it faintly alkaline; have the third exactly neutral. Heat them as in the last experiment, observing the coagulation temperature by means of a sensitive thermometer. Report results and conclusions.

157. Test the solubility in cold water of some serum albumin which has been dried at a low temperature. It dissolves slowly, showing that coagulation has not occurred. The solution will give the albumin reactions.

158. Place some of the thoroughly dry serum albumin in a dry test tube and let this stand for several minutes in a beaker of boiling water. After removal it will dissolve in cold water as before. Coagulation has not taken place, since for this the presence of water is necessary.

159. Show that a solution of serum albumin forms an insoluble compound when added to solutions of mercury, silver, or copper.

160. What would be the result of contact of such solutions with body cells rich in albumin, such as the cells of the gastric mucosa?

161. Does the serum albumin molecule contain the tryptophane radical? the phenyl radical? the hydroxyphenyl radical? more than one amino group? cystine?

Apply such tests as to answer the questions.

Globulins.

The globulins are distinguished from all the other simple proteins by being soluble in dilute solutions of

neutral salts, but insoluble in water. From this solution they are precipitated by diluting freely with water or by removing the salt by dialysis. They can, in this manner, be separated from the albumins which remain in solution. The globulins are, as a rule, mostly precipitated by saturating the solution with neutral salts, like magnesium sulphate or by half saturation by ammonium sulphate. They are coagulated by heating with water. By the action of dilute acids they are converted into acid metaprotein. A common example is myosin, which is found in muscle.

162. Prepare myosin from lean meat by chopping about an ounce finely, then stirring it well with cold water to remove the albumin. Filter through muslin, and repeat the treatment with water until it is white or nearly so. (How can it be proved that the filtrate contains albumin? Try it.) Squeeze out most of the water and treat the residue with a 10 per cent. solution of ammonium chloride. For thorough extraction it should stand several hours, but enough for testing can be obtained by stirring for five minutes. Filter through muslin, then through paper. The filtrate contains the globulin (myosin).

163. To a beaker full of pure water add a little of the solution. The myosin is precipitated. Why?

164. Heat some of the globulin solution. It is coagulated.

165. To 5 c.c. of the myosin solution add as much saturated ammonium sulphate solution. A precipitate of the globulin forms. Is it chemically changed?

166. Saturate 10 c.c. with magnesium sulphate. The globulin is precipitated, but dissolves again on dilution with water. Has it been changed chemically?

167. From results of last two experiments how would the poisonous effects of neutral salts seem to compare with those of the heavy metals? Why?

168. To the solution of myosin add enough hydrochloric acid to make it contain 0.2 per cent. After it has stood a few hours an acid albumin has been formed. If the change is complete it does not coagulate upon heating. If there is a coagulum it is some of the unchanged myosin. In this case filter it out and test the filtrate for the acid albumin by carefully neutralizing it with dilute sodium hydroxide. The acid albumin which is formed from the globulin by the dilute acid is precipitated.

169. Try the general tests for albuminous substances (xanthoproteic, biuret, etc.). The myosin responds to all.

170. **The Preparation of a Crystallized Vegetable Globulin.**—The edestin of the hemp seed, which is a globulin, will serve as an example. Digest about 10 grams of ground hemp seed for half an hour at 50° to 60° with about 50 c.c. of 10 per cent. sodium chloride solution; 2 to 3 c.c. of the solution if heated in a test tube should yield an abundant coagulum if solution is sufficient; then filter. Divide the filtrate into two parts. Dilute one part while warm with warm water until it begins to be turbid. Let it cool *very* slowly. Put the second part in a dialyzer and allow the salt to diffuse into the surrounding water, changing the water outside frequently. Explain the precipitation of the globulin by these two methods. In both cases microscopic crystals will be found, octahedral or tetrahedral in form. The more slowly they are formed the more perfect they will be.

171. Show that the crystals are insoluble in water but soluble in salt solutions. This solution precipitates on dilution with water and coagulates by heating. It also gives the biuret and the xanthoproteic reactions. The edestin is therefore a globulin.

172. Summarize the properties of globulins, experimentally demonstrated, in which they are similar to the albumins, also those in which they differ.

When foods containing albumins and globulins are cooked are they changed? Does it make any difference whether water is added or not? Answer from your experiments.

The Albuminoids.

The albuminoids are found in the insoluble form, mostly in the bones of the body or the parts which are used for protection. They resemble the simple proteins in possessing essentially the same chemical structure and giving many of the same products when they are decomposed, differing, however, in other respects. They are characterized by great insolubility in all neutral solvents, and are not easily attacked by the reagents which dissolve and decompose albuminous compounds.

Elastin.

Elastin occurs in the connective tissues,—in the cervical ligament (*ligamentum nuchæ*) very abundantly. It differs from most of the proteins in containing no sulphur, except possibly some that is loosely combined with the molecule. In the moist state it is very elastic; when dry it is hard and brittle. By the action of the digestive ferments it is decomposed into bodies called elastoses, similar to the albumoses. It gives the general reactions of the proteins.

173. Prepare elastin from the cervical ligament of an ox by cutting it into thin slices and boiling it for several days to remove the gelatin. Boil then with 1 per cent. sodium hydroxide for several hours, afterward with water. Repeat the boiling with 10 per cent. acetic acid; then let it stand twenty-four hours in 5 per cent. hydrochloric acid. Wash with water, boil with 95 per cent. alcohol, and extract with ether to remove the fat. For the complete removal of the latter more than a week may be required.

174. Try the tests used for sulphur in simple proteins, and see that it is not present. Test it for nitrogen. Try two of the protein color reactions.

Keratin.

The keratins are the chief constituent of the horny part of the epidermis, of hair, horns, nails, feathers, etc. They contain

a large amount of sulphur,—4 or 5 per cent.,—a part of which is so loosely united that it is set free by boiling water. It is owing to this sulphur that the salts of lead, silver, and some other metals act as hair-dyes, the sulphur uniting with the metal to form a dark colored sulphide. The keratin is not at all attacked by the gastric or pancreatic juices. It is decomposed when heated, giving the odor of burnt horn. It is insoluble in water, and gives the xanthoproteic and Millon's reactions.

175. Prepare keratin by boiling some horn shavings with water and then digesting them in succession in a dilute solution of pepsin containing 0.2 per cent. HCl, and a trypsin solution. Wash with water, alcohol, and ether.

176. Boil keratin with sodium hydroxide, filter, and test the filtrate for sulphur by lead acetate. It produces a black precipitate of lead sulphide.

177. Show that keratin responds to the xanthoproteic and Millon's tests.

Collagen.

This substance is found in the animal body in the connective and cartilaginous tissues, tendons, and bones. That from the bones was formerly called ossein. It is composed of the same chemical elements as the albumins, but contains a little more oxygen. It is insoluble in water, but by boiling with water it is converted into gelatin or glue. By the action of tannic acid collagen is changed to a form which does not putrefy. This is the action which takes place when leather is tanned.

Gelatin.

Gelatin may be considered as the hydrate of collagen, as it is formed by the union of collagen with water. It swells up in cold water and dissolves when the water is warmed. On allowing the solution to cool it gelatinizes,

or becomes a semi-solid. After it has been boiled a long time with water it is decomposed, and does not gelatinize on cooling, peptone being formed. The sulphur of gelatin is united in the molecule in a different manner from part of that of the albumin molecule, as is proved by its decomposition products.

Gelatin is decomposed by the gastric juice, giving products similar to those from albumin. It has been found, however, that it cannot take the place of the albuminous materials of food, though it is of value when used with them.

Gelatin does not give all the reactions of the simple proteins, although it does give the same results with some of them. Like the simple proteins, it gives a purple color with the biuret test; it is precipitated by picric acid, by mercuric chloride in the presence of sodium chloride and hydrochloric acid, by tannic acid in the presence of sodium chloride, and by saturation with ammonium sulphate. On the other hand, it is not coagulated by boiling; it is not precipitated by mineral acids, and it does not give a brown color when warmed with an alkaline solution of lead, as albumin does, the sulphur being apparently too firmly united to be split off and form lead sulphide. It does not give the xanthoproteic reaction when pure.

178. Prepare collagen from bone by dissolving out the mineral constituents with dilute hydrochloric acid (HCl , 1 part; water, 8 parts) for a week, or until they are flexible, then wash out the acid. Notice that the collagen is not soluble in dilute acid nor cold water. To remove all the albumins it may be necessary to soak awhile in 5 per cent. sodium hydroxide solution, then wash again.

179. Convert the collagen into gelatin by boiling it for half an hour or more with a little water. Notice that it gelatinizes upon cooling the solution, especially after standing.

180. Boil a portion of the solution for some time, and notice that it is thus decomposed, so that it will not form a jelly upon cooling.

181. Show that it is precipitated by tannic acid in the presence of sodium chloride.

182. Heat a solution of purified gelatin (not that just prepared from bone) with a solution of lead acetate in an excess of sodium hydroxide (127). Try the oxidation test for sulphur (125). Reconcile the two results.

With a solution of purified gelatin try the xanthoproteic, biuret, Millon's, and glyoxylic acid tests. Compare the results with those obtained with albumin and explain any differences.

What comparison is thus made between the gelatin and albumin molecules?

183. Show that gelatin is not precipitated by nitric or other mineral acids, but is by saturation with ammonium sulphate and also by mercuric chloride in the presence of HCl and NaCl.

184. Show that gelatin contains sulphur by heating with dry sodium carbonate in the reducing flame, then testing with sodium nitroprusside as in the case of albumin (131).

185. What are some of the animal food tissues, other than bones, containing an abundance of collagen? In what parts of these tissues is it found? What is the effect of cooking such tissues, as to change in their physical properties and chemical change in the collagen?

The Metaproteins.

These are called also acid and alkali albumins. They are formed from the simple proteins by the action of acids or alkalies. They are soluble in water which contains a small amount of acid or alkali, but are not soluble in neutral solution. Consequently they are precipitated when their solution is neutralized. They differ from the globulins by being insoluble in dilute salt solutions. Their solutions are not coagulated by boiling. They are named from the manner in which they are produced, and not according to their chemical reactions to litmus.

Acid Metaprotein.

186. **Acid Albumin.**—Prepare by adding dilute hydrochloric acid to a solution of egg albumin till it contains 0.2 per cent. of the acid. Allow it to stand at least an hour, at about body temperature, then filter and neutralize with very dilute sodium hydroxide, being careful not to add an excess, as this would dissolve the precipitated acid albumin. Wash the precipitate in water.

187. Notice that the acid albumin is soluble in acids, though insoluble in water. Make a solution in dilute hydrochloric acid and boil. It is not coagulated.

188. Make acid albumin by the action of concentrated hydrochloric acid, or acetic acid on serum albumin or egg albumin, warming if necessary. It is formed very quickly. Neutralize a portion with sodium hydroxide. It is precipitated. Show that it gives the xanthoproteic reaction and biuret reaction, though it is not precipitated by boiling.

Exactly neutralize the solution which is not coagulable by boiling, then heat the suspended precipitate to boiling. Try its solubility in dilute acid. Has it been coagulated?

Alkali Metaprotein.

189. **Alkali Albumin.**—Prepare from a solution of albumin by boiling with an alkali, like sodium hydroxide.

190. The solid alkali albumin can be obtained by adding concentrated sodium hydroxide, drop by drop, to the white of an egg, stirring continually. No more must be added after it has become gelatinous, as it will then dissolve. Wash the solid in cold water, in which it is insoluble, though it is soluble without difficulty in warm water. (It is known as Lieberkühn's jelly.)

191. Observe that the alkali albumin is soluble in dilute acids or alkalies. If the conversion is incomplete, complete solution does not occur in acids.

192. How can the solution be tested to determine whether some unchanged native protein remains?

193. Dissolve some of the solid substance in hot water or use the solution obtained from 189. Add a few drops of phenolphthalein, which gives a red color, showing that the solution is alkaline. Add slowly, dilute acid until the red color has just disappeared, when the solution will be neutral. The alkali albumin is precipitated. If, now, more acid is added, the precipitate dissolves if no unchanged albumin is present. It is, consequently, like the acid albumin precipitated by neutralizing.

194. Show that dissolved alkali albumin, like acid albumin, is not coagulated by boiling.

195. Show there is a cleavage of egg albumin during the formation of alkali albumin with a splitting off of the

loosely combined (cystin) sulphur by testing in the following manner :—

First heat the albumin solution with sodium hydroxide for several minutes. The sulphur which has been split off from the albumin molecule remains in the solution as sodium sulphide and does not discolor a piece of filter paper which is moistened with lead acetate solution and held over the mouth of the test tube. Upon acidifying the hot solution with hydrochloric acid the sulphur previously liberated is given off as hydrogen sulphide gas, which immediately turns the paper brown.

196. In the same manner as in the previous experiment test with lead paper, the gas from a solution of albumin when boiled with hydrochloric acid, as in the formation of acid albumin. No discoloration appears; that is, the albumin has not been decomposed to such a degree as in the last experiment.

197. How do the metaproteins compare in molecular size with the native proteins from which they are derived? Does the acid or alkali unite with the protein molecule?

Coagulated Protein.

The albumins and globulins may be converted into the coagulated form by heating with water or by the continued action of strong alcohol. This is insoluble in water, but can be dissolved by caustic alkalies or by heating with the strong mineral acids, being thereby converted into alkali or acid metaproteins.

Fibrin.

Fibrin is formed as a gelatinous mass when fresh blood coagulates. If the blood is beaten during its coagulation

the fibrin collects into elastic strings, and remains so as long as it is moist. It can be freed from the blood coloring matter by washing with water or a salt solution.

Proteoses and Peptones.

The peptones are the products of the digestive action of pepsin and other proteolytic enzymes upon the albuminous compounds. The proteoses are intermediate products between the albuminous compounds and the peptones. They may both be formed by the putrefaction of simple proteins, or by their hydrolytic decomposition by chemical agents.

The proteoses give the general reactions of the simple proteins, but they do not, like the former, coagulate on boiling. They are distinguished from the peptones by giving a precipitate with nitric acid or potassium ferrocyanide acidified with acetic acid. They are also precipitated by saturating their solution with ammonium sulphate or sodium chloride, then acidifying. They diffuse with difficulty through an animal membrane.

The peptones do not give any of these reactions, but respond to the general ones of simple proteins, especially the biuret test, where the resulting color is a reddish pink. They do not coagulate on boiling, and, unlike most of the other simple proteins, will pass through a parchment or animal membrane. They cannot be precipitated by ammonium sulphate or with potassium ferrocyanide. Tannin or alcohol precipitates them from their solutions.

A number of different classes have been described, two of the principal ones being those designated by the prefixes—*anti*, which resist the action of ferment and are not easily decomposed further; and *hemi*, which are more

easily decomposed. Thus we have antialbumoses and hemialbumoses, and antipeptones and hemipeptones. The other classes differ principally in their solubilities.

In the dry state the proteoses form an amorphous powder. The peptones also have an amorphous form, but are extremely hygroscopic, dissolving, to a resinous mass, in the water which they absorb from the air. Their taste is unpleasant.

198. Preparation of Proteoses.—Boil for twelve to fifteen hours about 10 grams of fibrin or coagulated egg albumin with three to four times its weight of 4 per cent. sulphuric acid. Keep the volume constant by the use of an inverted condenser or by the addition of water. The liquid turns violet, then brownish. If on testing a small sample by neutralizing with sodium hydroxide much acid albumin is precipitated, continue the heating until such a precipitate is no longer produced or is but slight. Then filter off any insoluble matter, neutralize with ammonium hydroxide, and saturate the liquid with ammonium sulphate. The proteoses are precipitated. Filter and wash them with a saturated solution of the last reagent. Then dissolve the proteoses in a little water, place this solution in a dialyzer, and dialyze until the outer liquid gives no reaction for sulphates with barium chloride. If the water is warm some antiseptic like thymol or chloroform must be used. The proteose reactions can be made with this solution from the dialyzer.

If the dry proteoses are desired, concentrate the solution to a small bulk and precipitate them with an excess of alcohol, washing with the same after filtration. Dry in a vacuum deiccator over sulphuric acid.

199. Dissolve some of the proteose in water and show that it is precipitated by potassium ferrocyanide in a solution acidified with acetic acid, avoiding an excess of the former.

200. Show that a solution of proteose in water is not coagulated by neutralizing nor boiling. Nitric acid gives a white precipitate which dissolves on heating and reappears on cooling. (Distinction from albumin and metaproteins.)

201. **Preparation of Peptone.**—Digest blood fibrin in a neutral solution with a watery extract from a chopped pancreatic gland at about body temperature. If it is allowed to stand many hours, add a few crystals of thymol to prevent putrefaction. Boil after solution has taken place, filter, concentrate by boiling, and saturate while boiling with ammonium sulphate to precipitate the proteoses. Filter these out, first by muslin, then by filter paper. The solution may be used for testing or the ammonium sulphate may be removed largely by evaporation and crystallization. The remainder can be removed by adding first barium hydroxide, then barium carbonate, and boiling, until a portion of the filtrate gives no precipitate with barium chloride.

202. Commercial peptone may be used for testing. Test the solution of peptones by adding first sodium hydroxide, then not more than two or three drops of copper sulphate (biuret test). A red or pink color is produced, which is characteristic of the peptones. If too much of the copper solution is added, the color is bluish.

203. Place some of the solution in a dialyzer and leave it an hour, then test the solution outside for the presence of peptones. They will be found to have passed through the membrane, although they do not dialyze rapidly.

204. Show that tannic acid precipitates peptones in a neutral solution.

205. Show that the peptones are not precipitated by potassium ferrocyanide acidified with acetic acid, as are

the proteoses if they contain none of the latter. The commercial peptone is usually a mixture of proteoses and peptones.

206. What are the relative molecular sizes of peptones, proteoses and native albumins? What proves it? Is it related to ease of coagulation by heat?

THE CONJUGATED PROTEINS.

This class of substances is more complex than the simple proteins. They can all be decomposed into simple proteins, on the one hand, and, on the other, organic compounds which are not proteins. Thus the simple protein is, in oxyhemoglobin, united with the hematin molecule; in the nucleins, with nucleic acid, etc. They can be considered, then, as unions of a simple protein with some other substance. Most of them are coagulated by boiling.

Glycoproteins (Mucins).

Glycoproteins are found in some of the secretions of the body, especially in those of the mucous membrane and saliva, and also as a constituent of the tendons and umbilical cord. Mucin is the most common example. In their composition they resemble the albuminous substances, but contain less nitrogen. Their characteristic property is that when boiled with a dilute mineral acid they are decomposed, giving two substances: simple protein and a compound containing little or no nitrogen and having the power of reduction, as is shown by its changing cupric hydroxide to cuprous oxide in an alkaline solution. By this they can be distinguished from all similar protein compounds. There are several varieties, although as yet they are not well differentiated from one another. The mucoids of the tendons are one class.

CLASSIFICATION OF THE SIMPLE AND DERIVED PROTEINS ACCORDING TO THEIR MOST IMPORTANT REACTIONS.

THE SIMPLE PROTEINS.

71

	Coagulable by Heat	Soluble in Water	Soluble in Salt Solution	Soluble in Acid or Alkalies	Soluble in Alcohol	Precipitated by Saturation with $(\text{NH}_4)_2 \text{SO}_4$	Diffusibility
Albumins . . .	+	+	+	+	—	+	—
Globulins . . .	+	—	+	+	—	+	—
Metaproteins	*	—	—	+	—	+	—
Proteoses . . .	—	+	+	+	—	+	+
Peptones	—	+	+	+	—	—	++

A knowledge of the above properties is necessary for the separation of these proteins as well as their identification.

* In absence of acid or alkali.

The glycoproteins are colloidal substances, insoluble in pure water but soluble in small amounts of dilute alkalies, such as calcium hydroxide. They are mucilaginous and can often be drawn out into threads. They are precipitated by the addition of acetic acid, if neutral salts are absent. They are not coagulated by boiling, but give many of the reactions of the albumins. Like the nucleoproteins, they are acid in reaction.

207. Preparation of Mucin.—Mince finely a submaxillary gland of an ox and extract it with water. Filter and add to the filtrate concentrated hydrochloric acid until the liquid contains 0.15 per cent. of acid, avoiding an excess. The mucin is at first precipitated, but dissolves again upon stirring. Then add two or three volumes of water, which will precipitate it. Separate it from the liquid by filtration or decantation, and repeat the dissolving and precipitation as before. Wash with water and, if the dry substance is desired, wash with alcohol and ether.

208. Try the solubility in calcium hydroxide or in very dilute sodium hydroxide, and precipitate from this solution by acetic acid.

209. Boil mucin for some time with dilute hydrochloric acid, and, after making the liquid alkaline, show by Fehling's test that there is a reducing body present.

210. Show that solutions of mucin in an alkali will give the biuret test.

211. What do the last two experiments prove about the composition and classification of mucin? How does it compare in solubility and means of precipitation with albumin? With alkali metaprotein? Does it differ in composition from a metaprotein?

Proteins Containing Phosphorus.

Nucleoproteins are compounds of one or more protein molecules with nucleic acid.

Lecithoproteins are compounds of the protein molecule with lecithins.

Phosphoproteins are compounds of the protein molecule with some other phosphorus compound than nucleic acid or lecithins.

Nucleoproteins, Including Nucleins.

The nucleoproteins occur widely distributed in the animal and vegetable tissues, particularly in cellular structures. They form one of the principal constituents of protoplasm. The nucleins (compounds of a single protein molecule with nucleic acid) are found united with an additional protein molecule as nucleoproteins.

Both nucleins and nucleoproteins have marked acid properties, and give the general reactions of the simple proteins, as might be inferred from their containing the protein molecule. They are coagulated by heat; alkalies dissolve them and acids precipitate them from this solution. With an excess of the acid the nucleoproteins dissolve with decomposition into nuclein and a protein; the nucleins are insoluble in dilute acids and usually in gastric juice. By hydrolysis they give purin bases, phosphoric acid, a carbohydrate or its derivative, and a protein or its decomposition products. Nucleins contain from 4 to 7 per cent. of phosphorus, and frequently iron. The iron in hemoglobin is probably derived from such compounds as these. One of the nucleins which appears to furnish iron for the synthesis of hemoglobin is hematogen, found in the yolk of the egg. The nucleoprotein

from this source can be decomposed by gastric juice, a part of the protein being digested and hematogen, a nuclein, left.

The iron in such organic combinations as nuclein does not respond to the ordinary chemical tests until the compound has been decomposed by chemical agents or other means. Reagents like ammonium sulphide and potassium ferrocyanide decompose it very slowly, whereas they act immediately upon the inorganic compounds of iron and not at all upon hematin.

Hematogen.

212. Prepare from the yolk of an egg. Shake the yolk in a wide mouth, glass-stoppered bottle with two or three times its volume of alcohol; allow it to stand and when it has settled pour off the alcohol. Repeat this operation twice, then extract in the same manner, or better in an extraction apparatus; with ether until the residue is white. Digest this in artificial gastric juice. The nuclein, hematogen, remains.

213. Dissolve a portion in ammonia. Test for iron by ammonium sulphide. At first there is no color, but after a time the solution turns greenish and, in twenty-four hours, black as the iron is gradually set free from the compound. In the same manner test with potassium ferrocyanide. The Prussian blue is formed slowly, differing thus from its production with the ordinary iron salts.

214. Add to the nuclein some hydrochloric acid, then, after neutralizing with ammonia, test for iron as before. The acid has decomposed the nuclein, so that the tests are obtained immediately.

215. **Preparation of Nucleoprotein from Yeast.**—In a mortar grind a cake of compressed yeast with as much water, 2 to 3 c.c. of ether and a pinch of fine sand, to aid in rupturing the cells. Dilute to about 100 c.c. with 0.5 per cent. sodium hydroxide, mix thoroughly and, after adding 2 to 3 c.c. of toluene as a preservative, let it stand over night to dissolve the nucleoproteins, stirring occasionally. Filter through a wet paper, and very

slowly add dilute hydrochloric acid while stirring until the nucleoprotein precipitates, leaving the liquid clear. Avoid an excess of the acid, as this would dissolve the nucleoprotein. Filter through a wet paper.

Hydrolyze the nucleoprotein and show the presence of its four components.

The hydrolysis is accomplished by boiling the nucleoprotein in a 100 c.c. Erlenmeyer flask with about 25 c.c. of 5 per cent. sulphuric acid. Add water to keep up its volume and continue the boiling at least an hour. If the liquid turns brown this is due to decomposition products, which can be neglected. Filter and take a quarter of the filtrate for each component, protein, purins, carbohydrate, and PO_4 .

- A. Test for proteins by at least two color reactions.
- B. Test for purins by making slightly alkaline with ammonium hydroxide, filtering if a precipitate appears. For the purin reagent use a silver nitrate solution into which enough ammonium hydroxide has been dropped to first precipitate silver oxide and then redissolve it by excess of ammonium hydroxide. A brown precipitate of silver purin compounds appears, very slowly if only a little purin is present.
- C. Test for carbohydrates by Trommer's or Fehling's test.
- D. Test for the PO_4 radical by acidifying with nitric acid, adding ammonium molybdate or molybdic acid solution and warming; a yellow precipitate appears.

216. What do the above experiments prove about the composition of the nucleoprotein molecule?

Nucleic (Nucleinic) Acids.

These occur combined with the protein molecule as nucleins in animal and vegetable cells, and are named from the tissues where they are found. The composition of the nucleic acids is shown by hydrolyzing them in the presence of a mineral acid, when they yield phosphoric acid, a carbohydrate (either a hexose or a pentose), one or more purin bases and, frequently, one or more pyrimidin bases. In acid solutions they precipitate albumin,

forming a compound which closely resembles the nucleins. They act as dibasic acids and easily form metallic salts. They give the xanthoproteic reaction.

Phosphoproteins.

Casein and vitellin are common examples of the group.

The phosphoproteins were formerly classed with the nucleoproteins, although they do not contain nucleic acid or lecithin, but phosphorus in another form, sometimes called paranuclein or pseudonuclein. No purin bases are present, but they may contain iron as well as phosphorus. Phosphoproteins are distinct acids, reddening litmus; they are insoluble in water or acids and form soluble salts with the alkalies. Where not too much of the alkali has been used in forming the salt the solution has an acid reaction to litmus. The phosphoproteins, therefore, act like dibasic acids. They can also liberate carbon dioxide from carbonates. When their alkaline solution is treated with an acid the phosphoprotein is set free as an insoluble substance, as is seen in the precipitation of casein from milk. Solutions of their salts do not coagulate on heating.

In general, being compounds of the simple proteins, the phosphoproteins respond to the same tests.

217. Preparation of Casein.—Dilute about 100 c.c. of milk with 400 c.c. of water, and precipitate the casein until the liquid above is nearly clear by adding acetic acid drop by drop, avoiding an excess. Filter and wash with water. If fat-free casein is desired, it must be extracted with ether in an extraction apparatus. For many tests this is not necessary. The casein can be to some degree purified by dissolving in dilute ammonia and reprecipitating with acid.

218. Test the casein for nitrogen and sulphur in the same manner that albumin was tested (123 and 127).

219. To about 5 c.c. of concentrated nitric acid in a small evaporating dish add a pinch of dry casein and boil in a hood, finally evaporating nearly to dryness. Dilute with about 10 c.c. of water, filtering if it is not clear, then add ammonium molybdate. A yellow precipitate, at once or after warming, shows the presence of the PO_4 radical.

220. Try the solubility of casein. It is insoluble in water, but soluble in alkalies. Show that its alkaline solution is not precipitated by heating. Add clear lime water; what is the milky solution? Test the solid substance with Millon's and the xanthoproteic tests; what do the results indicate? What radicals are present?

221. Demonstrate the acid nature of freshly precipitated casein by dissolving it in a very dilute solution of sodium carbonate. If not quite enough of the latter is used to produce a complete solution, and if the mixture is then filtered, the filtrate will have an acid reaction showing that the sodium-casein which it contains has some of the properties of an acid salt. Boiling causes no coagulation, but the casein can be set free as a precipitate if hydrochloric acid is added to strong acid reaction. (Compare with results of acidifying solutions of soap).

222. If the rennin ferment is at hand or can be prepared, test with it a solution of casein in lime water. The casein is changed into paracasein calcium (cheese) which is only slightly soluble in water.

223. How far are the phosphoproteins and the nucleoproteins similar in composition? What is their essential difference?

OUTLINE FOR THE SEPARATION OF THE MOST COMMON PROTEINS FROM THEIR SOLUTION.

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Look for metaprotein, phospho- and nucleoproteins. mucin, albumin, globulin, proteose, peptone and gelatin.
Try the reaction of the solution to litmus paper; make it exactly neutral with NaOH or HCl. Filter if there is a precipitate.

Precipitate. Look for metaprotein, mucin, phospho-
or nucleoproteins.

If the solution
was acid the
precipitate is
acid metapro-
tein.

Confirm by
solubility test.

If solution was alkaline, precipitate
can be alkali metaprotein, mucin,
phospho- or nucleoprotein.
Slightly acidify and filter.

Boil half
the precipi-
tate with
HCl; neu-
tralize.
Try Trom-
mer's test.
Mucin gives
a positive
result.

Precipitate. Look for albumin, globulin, proteose, pep-
tone, gelatin.

Half saturate with ammonium sulphate.

Precipitate. Look for
globulin, gelatin or
primary proteose.
Dilute with 30 to 40
volumes of water.

Precipi-
tate-al-
bumin.
Filtrate.
Look for
proteose and
peptone.
Saturate hot with
ammonium sul-
phate.

Filtrate. Look
for proteose and
peptone.
Precipi-
tate glo-
bulin.
Filtrate.
Look for
proteose and
gelatin.
Try Hop-
kins-Cole
reaction.

Precipi-
tate-
proteose
peptone.

Neg-
ative,
gela-
tin.

THE PROTEINS.

Although the separation of individual proteins is usually a matter of considerable difficulty, the identification of unknown ones may be a valuable aid in fixing their properties in mind. This should be done without mechanically following any outlined scheme. This table is given as a basis of such work, though it is much better to have the student devise a plan of his own for the purpose.

223a. Identify unknown proteins furnished by instructor.

FERMENTATION.

By fermentation we mean the decomposition of an organic substance into simpler and more stable molecules, the agent which causes the change being itself unaffected. The agents are living organisms or are formed by such organisms. The living ferments—such as the yeast plant or bacteria—are often called the organized ferments. They have the power of reproduction and are composed of cells. Their action is due to the enzymes which they form in the cell. The non-living ferments are known as the unorganized ferments or enzymes. They may be excreted by the organized ferments or secreted by living cells, which latter is the case with the digestive ferments. They are organic substances, non-reproductive, and act outside of the cell where they were formed. Hence the active agents in all fermentation are organic catalysts.

The enzymes of the animal cell exist in the cells in an inactive condition, called zymogens, but become active after standing exposed to the atmosphere or being brought in contact with certain chemical compounds. The enzymes contain nitrogen and, from their properties, may be identical with the proteins. They are non-diffusible and soluble in glycerol and in water. They can be mechanically removed, without decomposition, from their

solutions by forming precipitates therein, to which they adhere, also by saturating the solutions with ammonium sulphate. A low temperature stops their action, and they are all killed below 100° if moisture is present. Most enzymes act best at about 38° C. The ferment is not destroyed, but its action is stopped, by a large accumulation of its own products.

The organized ferments contain albumin, fat, cellulose, and some inorganic salts. They survive a high temperature better than the enzymes, but are killed at 100° except certain spore forms. Moisture is necessary for them to act.

As is the case with the enzymes, a sufficient amount of their products stops their further action. This is illustrated by the effect of alcohol upon the yeast plant.

224. Add a little yeast to a dilute solution of cane sugar in water and keep it for some time at the body temperature.

Test the solution with Trommer's test. The copper compound is reduced by the glucose and levulose which have been formed from the sucrose through its inversion by the yeast. If allowed to stand a long time the glucose is changed by the yeast to alcohol and carbon dioxide.

225. Would this indicate that the yeast contains more than one enzyme, or would a single one cause both chemical changes?

226. Stir a little compressed yeast into lukewarm water in a test tube, and after it has stood a few minutes, add a few drops of ether and mix thoroughly by shaking. Fill the rest of the tube now with dilute cane sugar solution and let it stand inverted for twenty-four hours in a warm place, as in 56. The alcoholic enzyme (zymase)

does not act in presence of ether, so that there is no alcoholic fermentation with a formation of carbon dioxide.

227. Test the liquid with Trommer's test. It responds to the test, showing that the inverting enzyme is not destroyed, but has decomposed the cane sugar as before, to glucose and levulose.

228. Separate the mucous membrane from the muscular coating of a pig's stomach; chop finely and allow to stand several hours with two or three times its weight of dilute phosphoric acid (1 per cent.). Filter, and to the filtrate, which contains the pepsin, add lime water until the reaction is alkaline. The calcium phosphate which falls carries down the pepsin with it. Filter and dissolve the precipitate in dilute HCl. Place in a dialyzer, changing the water outside frequently. The acids and salts diffuse out, leaving the pepsin inside the dialyzer, as can be proved by adding it to 0.2 per cent. HCl and seeing that it will digest fibrin.

229. Test a part of the precipitated pepsin which has been obtained with the calcium phosphate for nitrogen. This may be done, after washing with water, by drying the precipitate, still mixed with calcium phosphate, then mixing with twice as much soda lime and heating in a dry tube. The nitrogen is converted into ammonia, which may be recognized by the odor and by its action on red litmus paper.

230. To a part of the dialyzed pepsin solution obtained in 228 add finely powdered ammonium sulphate, stirring meanwhile as long as it dissolves. The pepsin is precipitated like the albuminous compounds. Filter, dissolve the precipitate in a 0.2 per cent. HCl solution, and show that it will digest fibrin.

231. Collect some saliva in a test tube, place the latter in a beaker of water, and raise the temperature of the water to 65° or 70° C. Keep it at this point for five minutes. Then let a little of it stand a few minutes with a starch solution, testing afterward with Trommer's test. No glucose is produced, the ferment, ptyalin, having been destroyed by the heat.

Among the enzymes which cause oxidation are those which aid in the union of free oxygen with oxidizable substances, and others which act only in the presence of hydrogen peroxide, from which they liberate the oxygen. Catalase is an example of the latter class.

232. Cut two small pieces from a raw potato, boil one for five minutes in a test tube, then let the two stand in the air. Note from the change of color that oxidation goes on in the juices of the unboiled piece, but that in the boiled sample the catalytic agent has been destroyed by the heat and there is no discoloration.

233. Shake about 10 grams of raw potato scrapings with 50 c.c. of water and filter. Heat half the filtrate to boiling (filtering again if it is not clear) and compare 5 c.c. portions of the boiled and unboiled,—

- (a) Color after standing several hours, or over night.
- (b) Action with a few drops of alcoholic guaiacum solution after several hours. (A blue color is a sign of oxidation.)
- (c) Action of 2 to 3 c.c. of 3 per cent. hydrogen peroxide on standing a few minutes.

234. If something is contained in the raw potato which is destroyed at boiling temperature, turns guaiac blue, and forms a brown oxidation product with some cellular constituent of the tissues in presence of air, how would you classify this agent? Where have you seen action of a similar oxidase elsewhere than in potato?

235. Collect a considerable quantity of saliva, and put it into two tubes. Quickly cool one nearly to freezing and warm the other to body temperature. Add to these an equal amount of starch solution previously brought to

the temperatures of the respective portions. Allow the action to proceed for five minutes, then raise them both to the boiling point to stop fermentation. Determine, approximately, the amount of sugar formed by applying to each the bismuth subnitrate test (51). There has been little or no fermentation in the cold liquid.

236. In the same manner cool another portion of the saliva and, after ten minutes, warm to body temperature and show by its decomposing starch that the ferment is not destroyed by the cold.

237. Make a list of the properties of enzymes so far illustrated by experiments.

238. **Preparation of Lactic Acid by Fermentation.**—In 150 c.c. of boiling water dissolve 30 grams of cane sugar and add about 30 mg. of tartaric acid. Let the solution stand two days, then add 40 c.c. of sour milk and about half a gram of old cheese. After the addition of 15 grams of zinc oxide, allow the mixture to stand ten days at a temperature of 40° to 50°, with repeated stirrings. At the end of that time heat to boiling, filter while hot, and allow to cool. Zinc lactate will crystallize out on cooling if the solution is sufficiently concentrated. If it is not it should be allowed to evaporate to a smaller volume. It may be purified by recrystallizing. The acid can be obtained by dissolving the lactate in water and decomposing by hydrogen sulphide gas. Filter off the zinc sulphide, evaporate the filtrate to a syrup, and after it is cold extract the acid by dissolving it in ether.¹ When the ether has evaporated the acid will remain. It may be preserved for testing in the gastric juice tests.

239. **Preparation of Butyric Acid.**—Make a mixture as for the preparation of lactic acid, or use a part of that mixture. Allow the fermentation to go on as before, but for three or four weeks. In that time bubbles of hydrogen and carbon dioxide

¹ Remember that the vapors of ether are extremely inflammable.

appear and, after removing the zinc, butyric acid is found in the ether. It can be identified by its acid reaction and characteristic odor.

THE SALIVA.

The saliva is a mixture of the secretions of the parotid, submaxillary, and sublingual glands with that of the glands of the membrane of the mouth. Its reaction is normally faintly alkaline to litmus. The mixed saliva is a colorless, more or less viscid liquid, often opalescent. On standing it deposits calcium carbonate as a film on the surface. Examined microscopically it is seen to contain epithelial cells from the membrane of the mouth, air bubbles held by viscid liquid, and salivary corpuscles which resemble the lymph corpuscles. Bacteria are abundant.

The normal mixed saliva contains

I. Inorganic	{ Carbonates Chlorides Sulphates Nitrites Sulphocyanates }	of	{ magnesium. calcium. potassium. sodium.		
II. Organic	{ Albumin. Mucin. Ptyalin.				

Nitrites and sulphocyanates (thiocyanates) are often absent. The latter are most frequently found in the saliva of smokers. Nitrites are formed by the bacterial reduction of nitrates. The ptyalin has the power to convert boiled starch into dextrin and maltose. It is able to penetrate the granule of the unboiled starch slowly. Its presence can be detected by mixing the saliva with about

ten times its volume of a solution of boiled starch, keeping it awhile at body temperature, and after a few minutes testing for sugar. The ptyalin acts best at about 38° , and is, therefore, not the same ferment as the diastase of malt, which decomposes starch most rapidly at a temperature of 55° . Ptyalin is destroyed by acids, even as dilute as the 0.2 per cent. hydrochloric acid of the gastric juice. It, however, acts for some time in the stomach before the acid penetrates the mass of food in a large enough quantity to stop the fermentation.

The secretion is influenced by the nervous system. It can be increased by mechanical means, like chewing paraffin or a piece of rounded glass in the mouth; by chemical action, such as touching the tongue with a crystal of tartaric acid or filling it with the vapor of ether or acetic acid, or by electrical excitation. In collecting saliva for testing it should be accomplished without trying to hasten its flow by suction with the tongue, as this increases the amount of secretion from the mucous membrane and so dilutes the secretion of the salivary glands.

The composition of the saliva is changed by certain pathological conditions. The amount is diminished in all febrile conditions, also in diabetes and often in nephritis. It is increased by the action of some medicinal substances, like the mercury compounds, pilocarpine, and others; also by anything which causes irritation or inflammation of the glands. Urea has been found in it abundantly during nephritis. The reaction becomes acid in fevers and in diabetes, and this sometimes happens also after long-continued talking.

240. Collect for examination some saliva by letting it flow into the mouth without swallowing. Excite the flow by chewing a piece of soft paraffin. Notice the indi-

cation of mucin in the viscosity, as well as the lasting foam after beating it with a glass rod.

241. Let a portion stand exposed to the air, and notice the separation of calcium carbonate as a white film or turbidity.

242. Why does the calcium carbonate precipitate, and what holds it in solution before exposure to air?

243. Test a portion for potassium sulphocyanate by adding to it a very dilute solution of ferric chloride. A red color indicates the sulphocyanate. This can be immediately decolorized by the addition of a few drops of mercuric chloride solution.

Equations for reactions?

244. *A.* Test for nitrites by adding to the saliva a few drops each of solutions of sulphanilic acid and alpha-naphthylamin in acetic acid. A pink color appears in a few minutes. This is Griess's test.

Griess's reagent is,—

Sulphanilic acid: dissolve 0.5 grams in 150 c.c. of 3 per cent. acetic acid, and

Alpha-naphthylamin: boil 0.1 gram with 20 c.c. of water and filter hot through a small filter from which nitrites have been removed by washing. Dilute with 180 c.c. of 3 per cent. acetic acid.

B. Determine the amount of nitrites by using 10 c.c. of saliva with 1 c.c. each of sulphanilic acid and alpha-naphthylamin acetate solutions. For comparison, treat in the same manner 10 c.c. of a standard solution containing 0.01 mg. of nitrite nitrogen.¹ After five minutes com-

¹ To make the standard nitrite solution dissolve 0.049 gm. of pure sodium nitrite in one liter of water. 1 c.c. contains 0.01 mg. of nitrite nitrogen. Dilute 1 c.c. to 10 c.c. for comparison with saliva.

pare the colors in equal sized tubes, or a colorimeter, diluting the darker one until equal volumes have the same shade. The relative amounts of nitrites in the two tubes are proportioned to their volumes after dilution. Calculate the amount of nitrite nitrogen in the saliva, expressing it in parts per million.

245. If Griess's test reveals no nitrites, or but a small amount, in the saliva, rinse out the mouth with dilute potassium nitrate solution (about 1 per cent.); then collect the saliva and test again. Explain result. Will nitrates react to Griess's reagent?

246. Test for mucin by adding to the clear saliva acetic acid drop by drop. The mucin separates in white, stringy flakes.

247. Test for albuminous substances by the xanthoproteic reaction after removal of mucin.

248. **Preparation of Mucin from Saliva.** Collect 25 to 50 c.c. of saliva and slowly mix it with about four times its volume of alcohol. Let it stand over night and decant off the liquid. If the dry substance is desired, filter and wash on the paper with alcohol, then with a little ether.

249. Try the solubility in dilute acids and in dilute alkalies.

250. Try the biuret reaction, Millon's reaction, and the xanthoproteic reaction.

251. Test a small portion to learn whether it causes reduction, like a reducing sugar.

252. Boil the rest of the mucin for ten minutes with 5 per cent. hydrochloric acid. After cooling, make it alkaline with sodium hydroxide, and test for the presence of a reducing substance.

253. What conclusions would you draw, as to the composition of salivary mucin, from the results of the last three experiments?

254. **Preparation of Ptyalin.**—Collect a large amount of saliva and acidify with phosphoric acid. Then add milk of lime

until the liquid has a faint alkaline reaction. The phosphoric acid is precipitated as calcium phosphate and carries the ptyalin down with it. Filter and allow the water to drain off without washing. Place the precipitate in a beaker and add not more water than the original amount of the saliva. Stir it well and filter. This removes the ptyalin from the calcium phosphate, and it goes into the filtrate. Add to the filtrate an excess of alcohol. A white precipitate will separate, which is ptyalin mixed with inorganic salts. To free it from these, dissolve in a little water and precipitate with absolute alcohol. Repeat this operation if necessary. Dry it over sulphuric acid.

255. Test the aqueous solution of purified ptyalin. It is not precipitated by nitric acid like albumin, nor does it give the xanthoproteic reaction. It can be precipitated after a time by basic lead acetate, the filtrate being without action on starch.

256. Try the reaction of saliva to litmus and to phenolphthalein. Normally it is alkaline with the former and neutral with the latter. Since alkaline salts affect litmus while they do not change the color of phenol phthalein, it is evident that here no free alkalies are present, but that the reaction is due to alkaline salts.

257. If these results are obtained with two indicators, how is it proper to describe the reaction of saliva?

258. In a test tube treat about 5 c.c. of 1 per cent. starch solution (made as in 8) with a few drops of saliva, drop in enough iodine solution to give a distinct blue color, and set it in water of body temperature. Notice the change of color from the blue first obtained through purple and red to colorless (the achromic point). Then the liquid will reduce Trommer's reagent. Hence the starch has undergone hydrolysis, passing through the dextrins to sugar (maltose).

259. Is it possible that the carbohydrate of the mucin molecule reduces Trommer's reagent instead of a sugar being formed from the starch? Proof?

260. Try the same experiment with unboiled starch. Sugar is formed only after a long time and in small amounts.

261. Try the effect of dilute acids by diluting 1 c.c. of concentrated hydrochloric acid with 100 c.c. of water, then adding to the saliva an equal volume of the dilute acid. This makes the acidity of the whole about the same as that of the gastric juice. After it has stood a few minutes let the acidified saliva act on boiled starch as before. No sugar is produced.

262. Test 3 to 4 c.c. portions of saliva for other inorganic constituents.

A. Acidify with nitric acid and add silver nitrate. Chlorides give white silver chloride.

B. After acidifying with hydrochloric acid, barium chloride gives a fine, white precipitate of barium sulphate if sulphates are present.

C. Acidify with nitric acid, add ammonium molybdate solution and warm; phosphates give a bright yellow cloud.

D. Acidify with acetic acid and show that calcium salts are present by the formation of insoluble calcium oxalate when ammonium oxalate is added.

263. Place 2 c.c. of saliva in each of seven test tubes, measured by a pipette. From a burette containing 0.1 per cent. HCl run into the separate tubes 4 c.c., 2 c.c., 1 c.c., 0.5 c.c., 0.2 c.c., and 0.1 c.c. The last tube receives no acid. Add to each tube 2 c.c. of starch solution. Let them all stand in water at body temperature. Observe the changes in appearance of the solution. At intervals remove a drop of liquid from each tube by the pipette and test it with a drop of iodine, making notes of the speed of digestion. When a blue color is no longer produced Trommer's test for maltose can be made. Calculate the concentration of acid in each tube. Report results.

Make application to starch digestion in the stomach.

264. Place about 4 c.c. of saliva in each of four test tubes. Add from a burette or cylindrical pipette containing 1 per cent.

sodium carbonate 1 c.c., 0.5 c.c., 0.25 c.c., and 0.1 c.c., respectively, warming with boiled starch in a beaker of water and noting results as before. The alkali retards digestion, but does not prevent it. Report time of reaching achromic point.

265. Try the effect of free and combined acid on ptyalin as follows: In each of two tubes mix about 4 c.c. of saliva with a few drops of a strong solution of albumin. Use for an indicator two or three drops of tropeolin, which turns red with free acid, but remains yellow if the acid is combined with a protein. To each tube add very dilute hydrochloric acid, to the first enough to produce a permanent red color, to the second not enough for this. Try their digestive power on starch as before. The trace of free acid has destroyed the enzyme; the acid combined with the albumin hinders digestion, but does not wholly prevent it. Would this be of any importance as regards gastric digestion of starch?

266. In each of a series of test tubes place 2 to 3 c.c. of saliva, add half as much of the starch solution and a few drops of the following antiseptics: Chloroform, sodium fluoride, salicylic acid, alcohol, mercuric chloride, copper sulphate. Let the tubes stand in water at 38°, occasionally removing a drop and testing this for unaltered starch with iodine solution. Since mercury salts form a compound with iodine, it will be necessary to use more of the reagent in this instance. Report time of reaching achromic point. After fifteen to thirty minutes try Trommer's test for maltose. The antiseptics do not prevent digestion except the metallic salts which unite with the albuminous substances.

THE GASTRIC JUICE.

The gastric juice, secreted by the glands of the stomach, differs from the other digestive fluids in having an acid reaction. It is a clear, thin liquid containing, as inorganic constituents, principally the chlorides and phosphates of the alkalies and of calcium and magnesium. There is more hydrochloric acid than can unite with the bases, and this must consequently be in the free state.

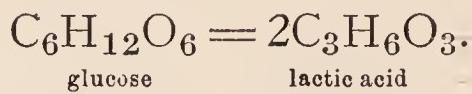
The most important of the organic substances are three enzymes, or unorganized ferments: pepsin, rennin, and a lipase.

The acidity of the gastric juice is caused principally by the free hydrochloric acid, but may be at times due to the acid phosphates, or to the organic acids: lactic, butyric, and acetic. The hydrochloric acid and acid phosphates are present in the normal juice. The lactic acid may be found in the first stages of digestion, especially when the food contains much of the carbohydrates, but is not normally found after digestion has proceeded more than half an hour. The acetic and butyric acids are not normally present.

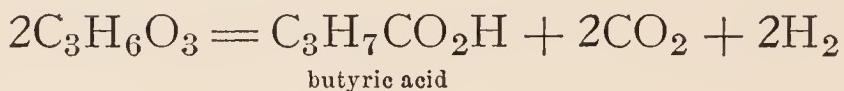
The free hydrochloric acid appears to be formed from the chlorides which are taken with the food. An acid is necessary for the digestion of the nitrogenous foods by the pepsin, and this is one of the offices of the hydrochloric acid, although one of its most important functions is the prevention of fermentation in the stomach. The mineral acids have antiseptic powers even in such dilution as that of the hydrochloric acid in the gastric juice; that is, from 0.4 to 0.5 per cent. Such a solution will, for several days, prevent putrefaction in animal matter, like chopped meat, which would otherwise soon commence to decay. It will also destroy the bacteria of many infectious diseases, though some of these are not affected when in the form of spores.

The effects of increased fermentation are seen in certain pathological conditions where the secretion of hydrochloric acid is diminished or stopped. They are especially noticeable in the case of food containing large quantities of carbohydrates. The sugar which is formed

by the saliva may be changed by the ferments into different acids:—



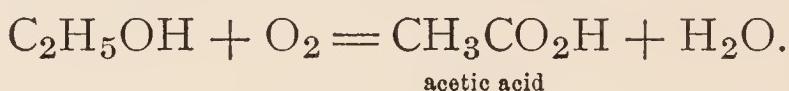
By further fermentation the lactic acid undergoes this change:—



The glucose may be fermented to alcohol:—



and this be converted to acetic acid:—



The so-called "heart-burn," which is one of the accompanying symptoms of gastric fermentation, is caused by the eructations of gas carrying the acids up into the throat, where they cause irritation of the mucous membrane. The methods of treatment based upon administration of hydrochloric acid, creasote, or other anti-septic substances are not for the purpose of removing the acids, but to stop the fermentation. Treatment with alkaline substances like magnesia or sodium bicarbonate neutralizes the acids, but does not prevent fermentation.

The acid phosphates may be present in the normal gastric juice. When present they increase the acidity of the juice or cause an acid reaction in the absence of free acids. An example is the potassium compound, KH_2PO_4 , which is found in the stomach after meat has been eaten.

The pepsin can be obtained from the gastric juice or mucous membrane of the stomach in a number of ways. Like most of the animal ferments, it can be extracted from the membrane by glycerol, and this glycerol solution

can be preserved for any length of time. If it is to be used immediately, water containing about 0.4 per cent. hydrochloric acid can be employed for extraction. If the dry substance is desired, it may be thrown down with finely divided precipitates of other substances, as is the case with many of the ferments.

The pepsin thus obtained is not the pure substance. It is a white or yellowish white, amorphous powder or scale when dried. It is hygroscopic in the air and has a slightly saline or acidulous taste, with no offensive odor. It is soluble in about 100 parts of water, but more easily on the addition of hydrochloric acid. Its molecular composition is unknown.

Pepsin is inactive in neutral or alkaline liquids, but in slightly acid fluids it dissolves coagulated protein compounds, with the formation of proteoses and peptones. It acts most rapidly with hydrochloric acid, though others may be used instead. The best concentration of acid for the purpose varies with the kind of material to be digested from 0.1 per cent. to 0.3 per cent. of hydrochloric acid. Pepsin from warm-blooded animals digests best at 38°. It is destroyed by heating the solution. Its action is hindered by the presence of the products of digestion, but if these are removed as fast as they are formed it will change to the soluble form many thousand times its weight of albuminous material.

The gastric juice acts only upon the nitrogenous constituents of the food. The simple proteins are first changed into acid metaprotein by the free acid; then this is decomposed by the pepsin and hydrochloric acid, forming proteose, which passes into peptone. Since the process is a continuous one, all these products may be found in the stomach at the same time, though in the first stages

the metaprotein is in excess and at the last the peptones. The connective tissues are digested by the gastric juice, though the proteolytic ferment of the pancreas does not dissolve them. The membranes which surround the fat cells are also dissolved, setting the fat free. Thus the food is changed into chyme, a pulpy mass which can be readily penetrated by the intestinal fluids. In the first stages of digestion the saliva may continue to act on the starchy materials, and during the first fifteen to twenty minutes there may be a formation of lactic acid, which disappears after this time. Milk is coagulated partly by the acid, partly by the rennin. The absorption of the peptones commences in the stomach, but the digestion is not completed here, the chyme passing through the pylorus into the intestine.

The second ferment of the gastric juice, rennin, is always present in human gastric juice under normal conditions. It exists in the mucous membrane in the form of a zymogen, which is sometimes inactive until it is set free by an acid. Hence if it is extracted by water it may not give its characteristic reaction. This is especially true in the case of birds or fish. This characteristic reaction is the coagulation of milk or casein in a neutral or faintly alkaline solution in which calcium salts are present. It does not give the albumin reactions when in a pure state. Rennin is more easily destroyed by heating its solution than is pepsin. The methods of obtaining it are similar to those employed with pepsin.

267. Preparation of Artificial Gastric Juice.—Separate the mucous membrane of a pig's or a dog's stomach from the muscular tissue. After rinsing it with water, chop it finely. Make a dilute solution of hydrochloric acid

by the addition of 1 c.c. of concentrated acid¹ to 150 c.c. of water. This will contain about 0.2 per cent. of the pure acid. Extract the pepsin from the chopped membrane by means of this dilute acid. To obtain a strong solution of pepsin it is best to let it stand for twenty-four hours in a cool place, though it will be found in the solution in a very short time. The rennin, which is dissolved at the same time, is destroyed by the pepsin on standing. Filter the liquid through muslin, then, if necessary, through paper. This solution can be used directly for digestive experiments or for preparing the purified pepsin.

268. Purified pepsin can be obtained by precipitating it with some substance which is thrown down as a finely divided precipitate. To effect this, neutralize the hydrochloric acid solution of pepsin in the preceding experiment and acidify with phosphoric acid, or extract the pepsin from the membrane with water acidified slightly with phosphoric acid. Precipitate the phosphoric acid solution by adding lime water or milk of lime until the liquid is slightly alkaline. Filter off the precipitate, which contains the pepsin mixed with calcium sulphate. Dissolve in water with the addition of hydrochloric acid. Remove the salts by dialysis. The pepsin does not pass through the membrane. A purer form is obtained by repeating the solution in acid and precipitation with lime water or by precipitating with alcohol before dialyzing. If the dry substance is desired, the drying must be at a low temperature. This may be done over sulphuric acid in a vacuum.

269. **Proof of the Existence of Zymogens.**—This is based upon the facts that dilute acids convert the zymogen into the enzyme and that dilute alkalies destroy the latter, but not the former.

Dilute 5 c.c. of the glycerol extract of the gastric mucosa from a recently killed animal in a test tube (A) with an equal volume of 0.2 per cent. hydrochloric acid. In another tube (B) dilute

¹ The U. S. P. acid containing about 32 per cent. HCl.

the glycerol extract with as much water. Warm both for fifteen minutes at 38° ; then add to each one-half of its volume of 1 per cent. sodium carbonate and warm at 38° for half an hour. Carefully neutralize them and add enough hydrochloric acid to make 0.2 per cent. Test for the presence of pepsin by a shred of fibrin. It is found in B, but not in A.

Where the membrane has been for some time exposed to the air before extracting the active enzyme may have been formed and consequently no difference be perceptible.

270. To make a pepsin solution which may be preserved, though it is somewhat impure, chop the mucous membrane finely, as in the previous methods, and after squeezing it in a piece of cloth to remove the water as far as possible, cover it with two or three times its volume of glycerol, and let it stand for a week. Filter it through a piece of muslin, pressing out the glycerol. This gives a permanent solution which can be used at any time for digestive experiments.

271. In each of a series of test tubes standing in a beaker of water at 38° place a little pepsin solution and a shred of fibrin. Then add to (a) lactic acid up to 0.8 per cent.; (b) oxalic acid up to 1 per cent.; (c) hydrochloric acid to 10 per cent.; (d) hydrochloric acid to 0.2 per cent.; (e) 0.2 per cent. sodium carbonate. Note the speed of digestion, tabulating results.

272. Make about 100 c.c. of artificial gastric juice by preparing a 0.2 per cent. solution of hydrochloric acid as in 267, and adding to this a small quantity of the glycerol solution of pepsin. The solution made directly from the gastric mucosa with the dilute acid can be used if it is fresh. (If a stomach cannot be conveniently obtained, 0.1 gram of scale pepsin in 100 c.c. of 0.2 per cent. acid can be substituted.) To this add 10 to 15 grams of washed fibrin. If this is not at hand, boiled egg albumin may be substituted for it, though in this case the digestion is

slower. Warm the mixture carefully to about body temperature and keep it at this point until the coagulated protein has dissolved. Notice that the edges first become transparent, then are dissolved. Too high heat will destroy the ferment. The fibrin should be nearly digested in half an hour. It may be left for several days without danger of putrefaction provided it contains 0.2 per cent. of free acid.

When the fibrin has nearly or quite disappeared, boil one-half the liquid to coagulate any unchanged protein, and filter. The filtrate contains the products of digestion: acid metaprotein, proteoses, and peptones. The proportion of each varies with the time of digestion. The amount of peptones is usually small until the pepsin has acted for some time.

Allow the second half of the digestion to proceed for a week before testing for peptones, adding hydrochloric acid if it is exhausted.

273. Precipitate the metaprotein by neutralizing carefully with very dilute sodium hydroxide. Filter it out and test as in 188.

274. From the filtrate precipitate the proteoses by saturating the solution with ammonium sulphate with the aid of heat. Filter and test the precipitated proteoses as in 199, and 200.

275. The filtrate from the proteoses contains the peptones. Test it according to 202 and 204. When the biuret test is made in presence of ammonium salts a large excess of sodium hydroxide must be used. It is advisable to add about a gram of the solid hydroxide to 10 c.c. of the solution to be tested. Why?

276. Report time of digestion and relative amounts of products formed as estimated from the bulks of their precipitates.

277. **Preparation of Rennin.**—Extract the chopped mucous membrane with 0.2 per cent. hydrochloric acid. Both rennin and pepsin go into solution. Stir the membrane well in the acidified water, but do not let it stand a very long time, as the rennin is digested in an acid solution by the pepsin. Neutralize carefully, add a small amount of magnesium carbonate, and shake well. The pepsin adheres to the carbonate and can be filtered out with it. Shake the filtrate with another portion of magnesium carbonate, filter, and repeat the operation until the pepsin is removed, which can be shown by the failure of an acidified portion of the filtrate to dissolve fibrin. The filtrate containing the rennin should coagulate milk in a few minutes.

278. In order to purify rennin, precipitate it with basic lead acetate and filter. Wash the precipitate, then suspend it in water, and acidify slightly with sulphuric acid. Filter, and to the filtrate, which contains the rennin, add a solution of stearin soap. The latter is decomposed by the acid, stearic acid being set free as an insoluble precipitate. This carries the rennin with it. Filter and place the precipitate, with a small amount of water, in a glass-stoppered funnel. Add ether, shake, and after the ether has separated above the watery solution, draw off the latter. The fatty acids from the decomposed soap have remained in the ether, leaving the rennin dissolved in the water.

279. Test the solution or a specimen of normal or artificial gastric juice for rennin by exactly neutralizing, then adding it to an equal volume of milk in a test tube. Place the tube in a beaker of water at body temperature. If rennin is present the casein will be coagulated in twenty to thirty minutes.

A solution of casein may be used instead of milk, but in order to make it coagulate a small amount of a calcium salt must be added.

For demonstrating the properties of rennin the com-

mmercial product, which is sometimes sold under the name of junket, may be employed.

280. Why neutralize the liquid when testing for the presence of rennin?

281. To 5 c.c. of milk add 1 c.c. of rennin solution and keep at body temperature. Note the time of coagulating.

Repeat the above, adding 2 c.c. of ammonium oxalate solution to the mixture of milk and rennin. This precipitates calcium from its solutions. How does it influence coagulation? Will the mixture now coagulate if calcium chloride is added?

282. Is the rennin, after it has been boiled, capable of coagulating milk? What does this show as to its nature?

283. Tabulate enzymes demonstrated in saliva and gastric juice, showing their substrates and products.

284. **Determination of Activity of Pepsin, U. S. Pharmacopoeia.**—Mix 25 c.c. (mils) of normal hydrochloric acid (36.47 grams per liter) with 275 c.c. of distilled water and dissolve 0.1 gram of pepsin in 150 c.c. of this liquid. Immerse a hen's egg, which is not less than five nor more than twelve days old and has been kept in a cool place, in boiling water during fifteen minutes. As soon as the egg has sufficiently cooled to handle it, remove the pellicle and all of the yolk; at once rub the albumin through a clean, dry, hair or brass No. 40 sieve; reject the first portion that passes through the sieve, and place 10 grams of the succeeding portion in a wide-mouthed bottle of 100 c.c. capacity. Immediately add 2 c.c. of the acid liquid, and, with the aid of a rubber-tipped glass rod, moisten the albumin uniformly. Again add 2 c.c. of the acid liquid, repeat the manipulation with the glass rod, and with gradually increasing portions of the acid liquid, until the total amount added measures 20 c.c. Thoroughly separate the particles of albumin from each other, rinse the rod with 15 c.c. more of the acid liquid, and, after warming the mixture to 52° C., add exactly 5 c.c. of the solution of pepsin. At once cork the bottle securely, invert it three times,

and place it in a water bath that has previously been regulated to maintain a temperature of 52° C. Keep it at this temperature for two and one-half hours, agitating the contents every ten minutes by inverting the bottle once. Then remove it from the water bath, pour the contents into a conical measure, having a diameter not exceeding 1 cm. at the bottom, and transfer the undigested egg albumin which adheres to the sides of the bottle to the measure with the aid of small portions (about 15 c.c. at a time) of distilled water until the total amount used measures 50 c.c. Stir the mixture well and let it stand for half an hour; the deposit of undissolved albumin does not then measure more than 1 c.c.

The relative proteolytic power of pepsin, stronger or weaker than that just described, may be determined by ascertaining through repeated trials the quantity of the pepsin solution, made as directed, required to digest, under the prescribed conditions, 10 grams of boiled and disintegrated egg albumin. Divide 15,000 by this quantity expressed in c.c. to ascertain how many parts of egg albumin one part of pepsin will digest.

Gastric Analysis.

In recent years the composition of the gastric juice and its variations in disease are being more and more thoroughly studied and the results of the observations made use of in clinical work.

With a little experience, the collection of the gastric juice for testing can be easily accomplished. In order to excite the flow a test meal should be given. Ewald's is rather simple, and consists of bread or rolls with weak tea without milk. A large amount of food rich in albuminous materials should be avoided, as the peptones resulting from its digestion interfere with some of the tests. With such a meal digestion is at its height in about an hour, and the collection should be made one to one and a half hours after eating. Hawk¹ recommends water as a gastric stimulant.

¹ Journal of Biological Chemistry, 1914, xix, 345.

The apparatus for withdrawing the juice is an elastic rubber tube about a yard in length, having a number of small perforations in the end, or, if a large opening, it should be on the side, to avoid injury to the mucous membrane by suction. The perforated end is passed slowly down the esophagus until it reaches the fundus of the stomach, known by the resistance to its further passage. The flow of the juice through the siphon is best started by pressure upon the stomach, the outer end of the tube being held lower than the stomach and over a collecting vessel. By this means the juice is not diluted.

While frequently an opinion as to the normality or abnormality of gastric metabolism is based upon the analysis of a single sample withdrawn from the stomach an hour or more after the test meal a more definite knowledge of this can be gained through the "fractional" examination as developed by Hawk and his co-workers. In this the Rehfuss stomach tube is used. This has a slotted metal tip on the lower end and a syringe at the upper for withdrawing the material to be tested. It is of such small diameter (5 mm.) that it can be left in the stomach without inconvenience through the whole cycle of gastric digestion. Small samples are withdrawn at definite intervals, and thus the changes in progress in the stomach can be carefully followed.

Before testing the juice it should be filtered through a plaited filter, keeping it covered to avoid loss of water by evaporation. If the gastric contents contain so much undigested matter or mucin as to interfere with reasonably rapid filtration, a square piece of moistened muslin can be substituted for the filter paper.

The chemical tests which are usually made upon the gastric juice are for:—

1. Reaction.
2. Hydrochloric acid,
3. Acid phosphates.
4. Organic acids {

Lactic.
Butyric.
Acetic (less frequently tested for).

5. Pepsin.

Others which are not so important, but the presence of which may sometimes be significant, are for :—

1. Starch.
2. Albuminous compounds.
3. Rennin
4. Blood pigments.
5. Bile.

Quantitative tests are valuable in the cases of :—

1. Total acidity.
2. Hydrochloric acid, free and combined.
3. Organic acids.

The reaction of normal gastric juice is, of course, acid. In some pathological conditions it becomes neutral or alkaline.

THE IONIC THEORY IN GASTRIC ANALYSIS.

The properties of solutions are best explained by the ionic theory, according to which the molecules in aqueous solutions are dissociated more or less completely into parts, or ions. The properties of solutions of acids are principally due to the hydrogen ion (H^+) and of alkalies, or bases, to the hydroxyl ion (OH^-). In pure water but one molecule in 10,000,000 is ionized; that is, the concentration of H^+ to H_2O is in the proportion of 1 : 10,000,000. This can be expressed (by logarithms) as 1×10^{-7} . In a neutral solution the number of hydroxyl ions is the same as that of the hydrogen ions.

For brevity, using the logarithmic figures without the minus sign, the concentration of the hydrogen ion in water, or a solution of the same degree of neutrality, is often expressed as $P_H = 7.0$. A concentration of the

hydrogen ion ten times as great would be $P_H = 6.0$; a concentration one hundred times as great as in pure water would be $P_H = 5.0$; one-tenth as great as in water would be represented as $P_H = 8.0$. It is to be noted that, in this nomenclature an increase in the logarithmic number signifies a decrease in the number of hydrogen ions.

In determining the degree of acidity or alkalinity of body fluids the most common method is to use *indicators*. These are substances whose color changes at a certain concentration of H^+ or OH^- . For example, when the concentration of hydrogen and hydroxyl ions in a liquid is the same (that is, in a neutral solution) litmus is purple. Or, when litmus gives a purple color to a solution we know that P_H is 7.0, or very nearly that. If the color is red, P_H is less than 7.0; if it is blue, P_H is more than 7.

If by acidity we mean the hydrogen ion concentration, it is evident that titration with an indicator will not indicate the true acidity. For the hydrogen of the acid molecule is only partly in the ionic form. This is particularly noticeable in case of the organic acids, which are relatively very little ionized. For instance, in a normal solution of acetic acid about four hydrogen atoms from a thousand molecules are in the ionic form.

If this solution is titrated with an alkali the four hydrogen ions will first be neutralized, then the remaining part of the thousand molecules will dissociate and their hydrogen ions will be neutralized.

The true acidity would consequently be four, the titratable acidity would be one thousand. It is important to bear in mind this distinction between true acidity of a solution and titratable acidity.

In physiological work the true acidity is most fre-

quently determined by the use of a series of indicators which change their colors at different, but definite, concentrations of the hydrogen ion. Solutions are prepared having a known concentration of the hydrogen ion. To these are added a few drops of indicators selected to show a certain shade of color for the concentration of the hydrogen ion of each solution. The unknown solution is treated in the same way with these indicators. When the shade of color is the same as that given by the known standard the concentration of the hydrogen ion (true acidity) is the same.

All indicators do not change color with the same concentration of the hydrogen ion (that is, the same degree of acidity). Thus, phenol phthalein changes from colorless to pink when there are more hydroxyl ions than hydrogen ions present, or the solution is slightly alkaline.

On the other hand, Congo red turns when the hydrogen ions are somewhat more numerous than the hydroxyl ions, or the solution is slightly acid.

In the following table is given a list of some of the indicators, with the points at which they change color.¹

	P_H
Phenolphthalein	8 to 9
Litmus	6 to 7
Sodium alizarine sulphonate.....	5 to 6
Congo red	4 to 5
Dimethyl aminoazobenzene	3 to 4
Tropeolin OO	2 to 3

285. To determine the total titratable acidity of the juice, filter it, keeping it covered to prevent, as much as possible, evaporation; then measure accurately 10 c.c. with a pipette and place it in a beaker. Add to this a few drops of an alcoholic solution of phenol-phthalein, which serves

¹ Salm. Zeitschrift für physikalische Chemie, LVII, 471.

as an indicator to tell whether the liquid is acid or alkaline during the determination, being red with alkalies and colorless with acids. Then add slowly from a burette a solution containing 4 grams of sodium hydroxide to the liter (this is one-tenth normal, or decinormal),¹ stirring continually, until the liquid is a faint pink color, which remains on standing a few minutes. Enough of the standard alkali has then been added to neutralize the acid substances present. Read off this amount from the burette. If no gastric juice is at hand, a solution for experimental purposes can be made of a mixture of the above acids after greatly diluting them.

For a short time after food has been taken hydrochloric acid may be wanting, or present only in traces in the gastric juice, without any pathological significance, but in one to three hours after a meal it should be found in larger amounts. It is then, under normal conditions, about 0.3 to 0.5 per cent. of the weight of the juice.

The common tests for the detection of hydrochloric acid cannot be employed in the case of the gastric juice, because the soluble chlorides which are usually present will respond to them. Special tests are used, most of which are based upon the fact that certain organic coloring matters are changed in color by a comparatively strong mineral acid, like hydrochloric, even in the dilute state, but are not affected by the weaker organic acids or acid salts. While these methods are not absolutely accurate, they are sufficiently reliable for clinical purposes when carefully performed.

286. Tests for Free Hydrochloric Acid.—Ascertain the color of the following reagents in neutral solution by adding a few drops of each to 3 to 5 c.c. of distilled

¹ It should be kept from long contact with the air to prevent conversion to the carbonate.

water in a series of test tubes; they are all used in gastric tests.

Methyl violet.

Tropeolin 00.

Congo red.

Alizarin sodium sulphonate.

Dimethyl-amino-azobenzene.

Phenol-phthalein.

Prepare a solution containing approximately one per cent. of hydrochloric acid. This can be done by adding 1 c.c. of the concentrated acid to about 40 c.c. of water. Add enough of this dilute acid to each of the tubes to bring the acidity up to 0.1 per cent. to 0.3 per cent., not more. The colors are then:—

Methyl violet, blue.

Tropeolin 00, red.

Congo red, blue.

Alizarin sodium sulphonate, yellow.

Dimethyl-amino-azobenzene, red.

Phenol-phthalein, colorless.

Pour the last three into small beakers or porcelain dishes and while stirring, drop in dilute sodium hydroxide until they are alkaline. The colors are then:—

Alizarin sodium sulphonate, violet passing into red.

Dimethyl-amino-azobenzene, yellow.

Phenol-phthalein, red.

287. Add to a few drops of 0.2 per cent. hydrochloric acid an equal volume of an alcoholic solution containing 2 per cent. phloroglucin and 1 per cent. vanillin. Evaporate to dryness in a porcelain dish on a water bath or by carefully warming over a flame. Avoid high temperature. A rose red color remains. (Günzberg's test.)

288. To a few drops of the HCl solution in a porcelain dish add a little of an alcoholic solution of 5 per cent. of resorcin and 2 per cent. of sugar. Evaporate to dryness and a red color appears. (Boas' test.)

Acid Phosphates.

The acid phosphates, such as K_2HPO_4 , may be normally present, but cannot perform the functions of the hydrochloric acid in digestion. Hence it is important to be able to detect them. They can be distinguished from free acids from the fact that they are not neutralized by calcium carbonate in the cold as the free acids are. When, therefore, a gastric juice is neutralized by adding to it finely powdered calcium carbonate, like precipitated chalk (which must itself be neutral), no acid phosphates are present, but the reaction was due to free acids. If the color of the litmus paper is obscured by the excess of calcium carbonate, this may be rinsed off with distilled water. If the reaction remains acid after calcium carbonate has been added, acid phosphates are present. Their amount can be determined by finding the total acidity, then adding calcium carbonate, filtering and determining the acidity of the filtrate. This latter is due to the phosphates. The difference between the two is the amount of the free acids.

289. Test the reaction of the acid phosphates¹ to litmus paper.

290. Show that they are not neutralized by calcium carbonate in the cold.

¹ Acid sodium phosphate, NaH_2PO_4 , can be prepared by adding carefully orthophosphoric acid to common sodium phosphate until it does not precipitate barium chloride. An excess of the acid must be avoided.

291. Show that the dilute free acids (both HCl and lactic) can be so neutralized.

292. Volumetric Determination of the Free Acids and Acid Phosphates.—To 10 c.c. of filtered gastric juice¹ add 5 c.c. of concentrated calcium chloride solution and a few drops of phenol-phthalein as an indicator, then $\frac{1}{10}$ normal sodium hydroxide until it is neutralized, when the color is a faint pink. The amount of alkali used corresponds to the total acidity of the juice. Then take 15 c.c. more of the filtered gastric juice and add about a gram of finely powdered calcium carbonate. Stir well and filter through a dry paper. Measure 10 c.c. of the filtrate into a small flask and by means of a rubber bulb or aspirator (not with the lungs) blow air through it to remove the carbon dioxide. Then add 5 c.c. of the calcium chloride solution and phenol-phthalein and neutralize with standard sodium hydroxide as before. Since the free acids are neutralized by the calcium carbonate, the sodium hydroxide used in this second determination corresponds to the acid phosphates, and the difference between the two to the free acid.

Subtract the number of c.c. of alkali used in the last determination from that used in the first. If lactic and volatile acids are present and have been determined, subtract also the number of c.c. required to neutralize them. The remainder has been used to neutralize the hydrochloric acid. Calculate the percentage of the latter, remembering that 1 c.c. of sodium hydroxide equals 0.00364 gram of hydrochloric acid.

Organic Acids.

Lactic acid changes a mixture of gentian violet and ferric chloride to a green or greenish yellow. None of the other normal or pathological constituents of the gastric juice appear to do the same or to interfere with the use of the above reagents in testing for the lactic acid.

¹ A mixture of HCl and phosphates may be used if juice cannot be obtained.

Lactic acid can also be detected in the gastric juice by the yellow color which it imparts to a solution of ferric chloride or to the amethyst solution which is produced by adding ferric chloride to carbolic acid (phenol), although glucose or alcohol gives a similar color.

It is generally unnecessary to determine the quantity of lactic acid. If this is desired, it can be done by measuring off 10 c.c. of the juice, diluting to about 100 c.c. and distilling off the acetic and butyric acids, which can be determined in the distillate. After the volatile acids have been removed by distillation until the liquid remaining is about 10 or 20 c.c., the lactic acid can be dissolved from the residue by shaking it six times with 100 c.c. of ether, separating the ether, then distilling it off. The lactic acid remains. Dissolve in water, add a few drops of phenolphthalein as an indicator, and see how much decinormal sodium hydroxide (4 grams per liter) is necessary to neutralize it. Each c.c. of the alkali corresponds to 0.009 grams of lactic acid.

293. The Gentian Violet Iron Test.—Use the two following solutions:—

No. 1. Saturated alcoholic solution of gentian violet,
0.2 c.c.

Distilled water, 500.0 c.c.

No. 2. Solution of ferric chloride (5 per cent.), 5 c.c.
Distilled water, 20 c.c.

In a porcelain dish place 1 c.c. of No. 1 and add No. 2 until a bluish violet color results. To this add the filtered gastric juice. Lactic acid changes the color to a green or greenish yellow. This is Arnold's test.

294. To about 10 c.c. of a 4 per cent. solution of phenol (carbolic acid) add a few drops of a solution of ferric chloride. Then dilute with water until the color is amethyst or reddish violet. Use this as a reagent for the detection of lactic acid. The color is changed to yellow by the lactic acid. This is called Uffelmann's test.

295. Test 0.2 per cent. hydrochloric acid in the same way. The solution becomes colorless; that is, hydrochloric acid gives no color of its own, hence would not conceal the lactic acid if both should be present. Try it.

296. Test the lactic acid with a solution of ferric chloride so dilute that it is scarcely colored. The yellow color is made stronger.

In stomach contents the test is more satisfactory if the lactic acid is removed from other substances by shaking 5 c.c. with 20 c.c. of ether in a separatory funnel, separating, and adding the ferric chloride to the ethereal solution. A greenish to yellow color appears.

297. Show that glucose or alcohol will give a yellow color with the above reagents (294 and 296).

Would the glycerol which is sometimes used to lubricate the stomach tube affect these reagents? How is it related chemically to glucose and alcohol?

298. Try the reaction of Congo red with 0.1 per cent. to 0.3 per cent. lactic acid and compare with hydrochloric acid reaction (286).

Butyric acid can be distinguished by its odor, which is that of rancid butter. It can be separated from its solution by shaking with ether. The acid is more readily soluble in ether than in water, and hence remains in the ether, and is perceptible when the latter evaporates. It can also be removed by distillation, as it passes off with the steam. The acetic acid is also volatile, and if present it distills with the butyric acid. If the steam is condensed the quantity of these volatile acids can be determined by the use of a standard solution of sodium hydroxide. Since they both have the same significance,

indicating fermentation, it is usually unnecessary to separate them.

299. Shake 10 c.c. of dilute butyric acid in a test tube with about 4 c.c. of ether. Pour off the ether and repeat the operation. Allow the ether to evaporate, *away from lights and fires*, and notice the odor of the acid which remains.

Many methods have been proposed for determining the amounts of acids which at times are found in the gastric contents. Toepfer's is the one which is probably the most commonly used.

300. **Toepfer's Method for Determining the Free and Combined HCl.**—Into each of three beakers (*A*, *B*, and *C*) measure with a pipette 5 c.c. of gastric juice. Titrate each of them with decinormal NaOH (4 grams to the liter), using, as indicators, in *A* phenol-phthalein, and adding the alkali until the liquid is a faint red; in *B* 3 or 4 drops of a 1 per cent. solution of alizarin sodium sulphonate in water. The NaOH must be added until the liquid is a violet color. To get the exact shade make a standard for comparison by adding to a 1 per cent. solution of common sodium phosphate, Na_2HPO_4 , the same proportion of alizarine sodium sulphonate as was used in the test, and comparing them in equal sized test tubes. With *C* the indicator is 3 or 4 drops of a 0.5 per cent. alcoholic solution of dimethyl-amino-azobenzene. HCl gives a red color with this, and the NaOH is added until this disappears and the color changes to yellow.

To interpret the results we should remember that of the indicators used the phenolphthalein is colorless in the presence of all of the acid reacting substances of the gastric juice. *A*, therefore, represents total acidity. The

alizarine sodium sulphonate reacts with free hydrochloric acid, with organic acids, and with acid phosphates; that is, with everything except combined hydrochloric acid. Consequently $A-B$ represents the acidity due to the hydrochloric acid combined with protein, the so-called *organic* or *combined* hydrochloric acid. Dimethyl-aminoazobenzene is affected only by free hydrochloric acid, and this is shown in C . $B-C$ gives the acidity from the acid phosphates, plus the organic acids.

Calculate from the amounts of sodium hydroxide used the percentages of each of these, remembering that one c.c. of the alkali contains 0.004 gram and neutralizes 0.00364 gram of HCl. The amounts of acid phosphates and organic acids must be expressed in terms of an equivalent amount of HCl.

In clinical work instead of expressing acidity in terms of per cent. of hydrochloric acid, it is more often given in terms of the number of cubic centimeters of decinormal sodium hydroxide required to neutralize 100 c.c. of the gastric juice. For example, if in Toepfer's determination 3 c.c. of the decinormal alkali were necessary to neutralize 5 c.c. of the juice the acidity would be said to be 60.

301. Get samples of gastric juice, natural or artificial,¹

¹ If natural gastric juice cannot be obtained by the use of the stomach tube from students or from a hospital, an artificial substitute can be prepared by mixing hydrochloric, butyric and lactic acids and monosodium hydrogen phosphate in amounts of 0.1 per cent. to 0.4 per cent., not more than 0.5 per cent. in total acidity. If the organic hydrochloric acid is desired, add a little pepsin and a protein, like fibrin or albumin, and let it stand at 38° for an hour; or add commercial peptone to 0.2 per cent. hydrochloric acid and let it stand until the acid reaction has nearly or quite disappeared.

from the instructor, determine total and fractional acidities, and report results. -

Other Gastric Tests.

The presence of pepsin in a solution like the gastric juice is best detected by trying its digestive power on fibrin. Unless the liquid contains a proper amount of acid it must be acidified so as to contain about 0.2 per cent. of hydrochloric acid. The rapidity of digestion can be best perceived by coloring the fibrin dark red with a solution of carmine in ammonia. This coloring matter is insoluble in water, but is set free, coloring the liquid, as the fibrin dissolves. The depth of color denotes the amount digested. The colored fibrin may be kept on hand for any length of time by pressing out most of the water, then preserving in glycerol or ether.

302. Stain to a deep red some shreds of washed fibrin with a solution of carmine dissolved in ammonia. After washing with water, place these in several test tubes; add to each specimens of natural or artificial gastric juice, containing different amounts of pepsin, including one with none. If they are not sufficiently acid, make them so. Set the tubes in a beaker of water of about body temperature and notice the setting free of the color as the pepsin acts.

The rennin is detected by neutralizing and testing with milk for the power of coagulation as in 279.

The test for starch and its first decomposition product, dextrin, is iodine dissolved in potassium iodide. According to von Jaksch, neither of these remains in the stomach in normal digestion after the first hour, though they may be present as long as that when there is an excess of acid or deficiency of ptyalin in the saliva.

The albuminous substances and their digestive products can be detected by the methods given in 273, 274, and 275, and by the tests described for simple proteins.

The gastric juice never normally contains blood, but this is sometimes found in it or in the vomited material in cases of chronic ulceration of the stomach or after poisoning by the corrosive or strongly irritant poisons. The hemoglobin is usually decomposed and the hematin which results gives a dark brown color to the juice. It is best identified by the hemin test (360).

Occult blood (that is, blood in such minute amounts that macroscopically it is not evident) can be detected by the benzidine reaction (356) after a meat-free diet.

303. Mix about 1 c.c. each of gastric contents, 3 per cent. hydrogen peroxide, and a saturated solution of benzidine in glacial acetic acid. If the blood pigments are present a blue color appears.

304. To test gastric contents for bile, saturate about 10 c.c. of the filtered liquid in a test tube with ammonium sulphate crystals by shaking. Then add 2 to 3 c.c. of acetone and mix by turning the corked tube gently, but not shaking violently. The bile pigments are dissolved by the acetone. When this has risen to the top, pour it into another tube and add a drop of yellow concentrated nitric acid. A green color appears.

305. **Determination of Peptic Activity** (Mett's Method).—This is based upon measuring the length of a column of coagulated albumin digested in a narrow glass tube.

Hold a short piece of clean 5 to 6 mm. glass tubing in a Bunsen flame, and when it is soft draw it out so that it is 1 to 2 mm. in internal diameter and 20 to 30 mm. long.

Separate the white of a fresh egg from the yolk, stir it and strain through muslin. Suck up into the small tube the egg albumin, then lay the tube horizontally in a large dish of hot water (at 85° is best, as the albumin is then soft-boiled). If the ends are dipped in sealing-wax or paraffin they can be kept some time.

To make the determination make sections of the filled tube 1.5 to 2 cm. in length by marking with a file and breaking. Use only sections which have ends broken off at a right angle. Keep two of these tubes in sample of gastric juice in an incubator at 38° for twenty-four hours. Measure the length of albumin digested with a millimeter scale. In normal human gastric juice it will be 2 to 4 mm.

306. Sahli's Test for Digestive Function of the Stomach.— This is a method for trying digestibility of catgut, which is indigestible in the pancreatic juice of the intestine. The catgut is used to tie a rubber sack which contains methylene blue. When it is digested the sack opens, the blue is absorbed and appears in the urine.

Make a small pill or mass with 25 to 50 mg. of methylene blue and sufficient *ext. glycyrrhizæ*; lay it on a small piece of thin rubber dam, twist up the edges of the rubber to make a sack, and tie the mouth tightly with several turns of thin raw catgut which has been previously softened with water. Test the sack in a beaker of water; it should sink without coloring the water. Swallow the sack with the noonday meal, and examine the urine hourly after four hours until bed-time, then the next day. The presence of methylene blue is shown by a greenish blue color. Very small amounts may be shown by boiling the urine with one-fifth its volume of glacial acetic acid, when the greenish color will appear. Normally 18 to 20 hours is sufficient time to elapse for this.

It is occasionally desired to test the rapidity of absorption from the stomach. This can be accomplished by determining how long a time is necessary for potassium iodide to pass from the stomach through the circulatory system into the saliva. About 5 grains of the iodide is taken in a capsule or in water, in the latter case care being taken to thoroughly rinse the mouth afterward. The saliva is collected and tested every minute after the fifth. Place a little upon a paper dipped in starch-paste and dried, or add it to a few drops of starch solution. Then add a few drops of a solution of calcium hypochlorite (chlorinated lime) to set free the iodine, which colors the starch blue. It should appear in the saliva in from eight to fifteen minutes. If the urine is tested in the same manner there should normally be a positive reaction in 15 to 30 minutes.

The clinical tests for hydrochloric acid given above are open to some objections. Many are interfered with by large amounts of albumin and peptones. If they fail in the tests of a gastric juice the biuret test should be tried, and if any of these substances are present they should be precipitated by a 10 per cent. solution of tannic acid and, after filtering, the liquid should be again tested for hydrochloric acid. There is also a limit to the delicacy of the tests, so that very minute amounts of free acid may not be detected. These are so small when they cannot be thus detected that the condition may be considered pathological.

Methyl violet is turned blue by about $\frac{1}{3}$ of a milligram of acid. Tropeolin 00 is of about the same delicacy. Congo red is somewhat affected by the organic acids if they are not very dilute. The test paper made from this has the advantage of easy portability and that it can be preserved and also that something can be judged from the imparted color of the amount of acid present. With a large percentage it becomes a blue black, and with a small amount a lighter blue. Dimethyl-aminoazobenzene likewise responds to organic acids if they are in considerable amounts. Phloroglucin and vanillin make a sensitive reagent, it being possible to detect with it 1 milligram of free acid in 10 c.c. of juice.

The lactic acid test—with phenol and ferric chloride—is also in some cases uncertain. When it fails, however, the acid is not present. Some common substances, like sugar and alcohol, give the same results as the acid. When it is suspected that this is the case, the liquid should be shaken with ether to dissolve the acid, and the ether, after separation from the water, be evaporated to dryness. Dissolve the residue in a little water and test it for lactic acid. Arnold's test is more reliable.

307. Prepare a table of the reagents used for gastric hydrochloric acid giving (a) the color of the original reagent, (b) the color given by the acid, (c) the sensitiveness and reliability of each.

The results obtained from chemical testing of gastric juice or vomited material are often of great aid in diagnosis. The presence of the organic acids—lactic, butyric, or acetic—more than thirty minutes after taking food

indicates fermentative action, usually due to a deficiency of hydrochloric acid. After the same time a failure or excessive amount of hydrochloric acid can be considered pathological. It may be absent in acute or chronic dyspepsia and in chlorosis. It usually is not found, or is present in only small amount, in carcinoma of the stomach. With dilatation of the stomach caused by stenosis of the pylorus there is often an hyperacidity, more than 0.5 per cent. of the acid being present. With gastric ulcer the acidity is often high.

308. Make analyses of specimens of gastric juice furnished by the instructor, handing in written reports on the presence or absence of:—

1. Free hydrochloric acid.
2. Acid phosphates.
3. Lactic acid.
4. Butyric acid.
5. Pepsin.
6. Coagulable protein.
7. Non-coagulable protein.
8. Blood.

Also quantitative determination of:—

8. Total acidity.
9. Free hydrochloric acid.
10. Combined hydrochloric acid.
11. Organic acids and acid phosphates.

In each case include in the report a full explanation of the results as showing to what degree the juice is normal or pathological.

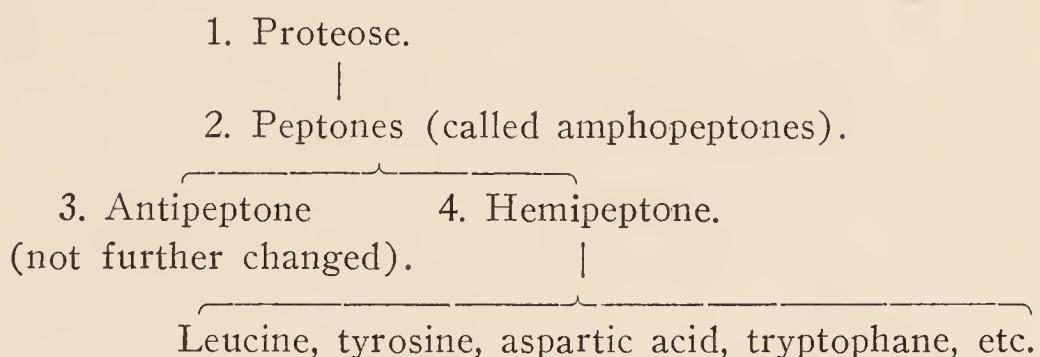
PANCREATIC AND INTESTINAL DIGESTION.

The fluid secreted by the pancreatic gland contains at least four enzymes which aid in the digestion of food: a protease, trypsin, which decomposes the proteins; a lipase, steapsin, which acts upon the fats; an amylase, amylopsin, which converts starch into maltose; and rennin, a casein coagulating ferment. These ferments occur in the gland in the form of inactive zymogens, but are changed into the active form a few hours after death, or by the action of chemical agents like traces of calcium salts or of acids. Trypsinogen, the zymogen of trypsin, is activated by enterokinase, a substance secreted by the mucosa of the intestine. The normal pancreatic juice is alkaline from the presence of sodium carbonate, though the aqueous extract made from the gland for demonstrations may be acid from the presence of sarco-lactic acid.

The trypsin dissolves fibrin and other coagulated simple proteins, but differs from pepsin in that it acts in a neutral or weakly alkaline medium. With trypsin the fibrin does not swell and become transparent before dissolving, as is the case with pepsin, nor is acid albumin formed as the first stage of digestion. The principal decomposition products of fibrin by the action of trypsin are proteoses and peptones; then the peptones are broken down by erepsin through the polypeptid stage (consisting of molecules containing a small number of amino acid radicals) to leucine, tyrosine, aspartic acid, and other amino acids. Tryptophane (indol alpha-amino-propionic acid), which gives a reddish violet precipitate with bro-

mine water or chlorine, is one of these. The antipeptones are not affected by the trypsin. The hemipeptones are decomposed. In normal digestion a relatively small proportion of the peptones is broken down by the trypsin.

The substances produced from simple proteins by the action of trypsin are:—



Leucine (alpha-amino-isobutyl acetic acid),



and tyrosine (para-oxy-phenyl-alpha-amino-propionic acid),



are formed in the decomposition of protein substances by putrefaction as well as in the normal processes of digestion, and hence they may be found as a result of pathological processes where there is a destruction of the proteins. Leucine crystallizes in the form of thin plates, which are usually, when impure, grouped together into round knobs or balls. These can be recognized by the aid of the microscope. (Plate II, 12, b.) In the impure state tyrosine forms aggregations of crystals which resemble those of leucine, but when purified it appears as fine silky needles often gathered into sheaves or balls. (Plate II, 12, c.) Tyrosine requires for its solution 2454 parts of water at 20°. Leucine can be

dissolved in 27 parts of cold water. This affords a means of separating them.

309. In a series of test tubes place a small amount of pancreatic extract, prepared as in 310. Boil one (*A*) ; acidify the second (*B*) with hydrochloric acid ; make the third (*C*) slightly alkaline with sodium carbonate ; make the fourth (*D*) neutral if it is not already so. Place in each a shred of fibrin and set the tubes in water at 40°. Let a fifth tube (*E*) be made alkaline and stand with fibrin in cold water. Note the change in the appearance of the fibrin and the speed of digestion. If the tubes are to be left for a long time putrefaction can only be prevented by the use of some antiseptic like chloroform water (chloroform, 5; water, 1000) or thymol.

Is solution here caused by an enzyme? Proof? What conditions most favor it?

310. Prepare an artificial pancreatic juice by extracting a finely chopped pancreatic gland of a hog or ox or dog with lukewarm water. It is better to wait an hour or two after killing the animal, in order to allow of the formation of the active ferment, or to use 0.2 per cent. salicylic acid solution for the same purpose; this also prevents putrefaction. Chloroform water gives an extract with marked proteolytic properties; alcohol gives a strongly lipolytic and amylolytic solution. Filter the extract through cloth and add to the filtrate a little chloroform or thymol to prevent putrefaction, which soon occurs without an antiseptic. Test three portions of the extract for the digestion of protein, fat, and starch, respectively.

A. Protein Digestion.—Place in about 100 c.c. of the liquid 10 to 15 grams of fibrin or of hashed lean beef,

make slightly alkaline with sodium carbonate, and allow it to stand at about body temperature until it has all dissolved. The products obtained will depend upon the time. If the digestion is stopped too soon they will be largely proteoses and peptones, but later there will be more of the leucine and tyrosine. The best results will be obtained by digesting for several hours, or as much as a day.

When digestion is well advanced test a small portion of the liquid by adding bromine water drop by drop, avoiding an excess. A reddish violet precipitate shows the presence of tryptophane, which only appears after the peptone molecule has commenced to break down. It is destroyed by an excess of the reagent.

Filter a second portion and cautiously add to the filtrate acetic acid until the reaction is neutral. Boil, and filter if the liquid is not clear. To this filtrate add a few drops of sulphuric acid, then crystals of ammonium sulphate until it is saturated. The precipitate of proteoses can be filtered out and the proteose reactions tried. The filtrate from the proteoses contains the peptones. Try the biuret test, which should give a pink color. On account of the presence of ammonium sulphate a large excess of the alkali must be employed; it may be more convenient to add this in the solid form.

Let the remainder of the original solution digest for a week in the presence of thymol or chloroform as a preventive of putrefaction, then concentrate the solution to a small bulk and precipitate the proteins from it by the addition of about twice the volume of alcohol. Filter these off and evaporate the alcohol, or distill it if there is a large quantity. Concentrate the liquid on the water bath to a thin syrup, then let it stand until the tyrosine

crystallizes out. Examine the form of the crystals under the microscope. If there is enough of the liquid to filter, remove the tyrosine and let the leucine crystallize from the filtrate. If there is only a small amount they can both be identified with the microscope. Sketch these and hand in the results. If there is a sufficient quantity they may be tested by the tests given in 319, 320 and 321. Re-crystallization often gives better crystals.

B. Fat Digestion.—Steapsin, the second ferment of the pancreatic juice, splits the fats into glycerol and the fatty acid with which it was united. The acid which has been set free in this manner unites with the carbonate of sodium which is present in the intestine; forming the sodium salt (a soap), and this serves to emulsify the rest of the fats by surrounding the globules with such a coating that they are not able to unite into a large mass. The sodium carbonate is not able to decompose the fat-molecule or to form a soap with the acid until the latter has been set free by the ferment, nor will it emulsify the fat if there is no fatty acid present, but a fine emulsion is produced after the decomposition.

The fat-splitting ferment is easily destroyed by acids; hence it may not be found in a gland which has been kept until it has an acid reaction.

311. Make a watery infusion of the pancreatic gland, as in the trypsin digestion. If it is not already exactly neutral, make it so. Add to it in a test tube a few drops of some neutral fat, like olive oil, or cottonseed oil, and let it stand for half an hour in a beaker of water at about 38° , shaking occasionally to keep the two liquids well mixed. Then test it with a piece of blue litmus paper. It will turn the paper red from the fatty acid which has been set free. Write equation for the reaction.

312. Mix 10 c.c. of cream or rich milk with a little powdered blue litmus, shaking well until an even blue color is obtained. Divide in two test tubes. To one add boiled pancreatic extract, to the other unboiled, and keep at a temperature of 38°. The former remains blue, the latter becomes red from the cleavage of the butter-fat into fatty acids and glycerol.

313. In each of two test tubes place 5 c.c. of water, 2 c.c. of ethyl butyrate and enough finely powdered litmus to give a blue color. To one add 3 to 4 c.c. of boiled neutral pancreatic extract, to the other the same amount of unboiled extract. Let them stand in water at 38°. Note the odor and color; interpret and write equation for the result. How far is this change similar to fat digestion?

314. Shake, in a test tube of water, to which a few drops of sodium carbonate have been added, a few drops of olive oil which does not contain free acids. (Since the oil easily becomes decomposed on standing, it may be necessary to remove the free acids by first shaking with very dilute sodium hydroxide solution and ether. Separate the ether from the water, wash well by shaking it a number of times with pure water, and allow it to evaporate at a gentle heat away from a fire or lamp. The alkali has united with the free acid to form a soap, and this has been washed out by the water, leaving the fat neutral.) If the oil is neutral it will form no emulsion with the carbonate. Add now a drop of a fatty acid like oleic acid and shake. A fine emulsion is formed immediately. Explain.

315. If a bottle of rancid oil is at hand, shake it with a weak solution of sodium carbonate, and notice that it contains enough of the free acids to form an emulsion at once.

C. Starch Digestion.—The third ferment of the pancreas, amylopsin, converts starch into maltose as the ptyalin does, except that its action is more energetic. Thus it acts upon raw starch which the ptyalin will do only slowly.

316. Treat boiled and unboiled starch in two test tubes with a small amount of the watery solution of the

pancreatic ferments made as before. Test for maltose by a reduction test. Notice that it is found with the boiled starch in a few seconds; after a longer time with the unboiled.

D. Action of Rennin.

317. Take 3 to 4 c.c. of the neutral pancreatic extract in each of two test tubes. Boil one; after it is cool add 10 c.c. of fresh milk to each. Let them stand in water at 38° and interpret the results.

318. To prepare leucine and tyrosine in larger quantities, take of white horn-shavings 2 parts and boil for twenty-four hours with 13 parts water and 5 parts of concentrated sulphuric acid, adding water as it evaporates. Dilute with water, and while warm neutralize with milk of lime. Filter, boil the precipitate several times with water, and filter in order to remove all the leucine and tyrosine. Unite these filtrates, and, after concentrating them by boiling, precipitate the calcium by the addition of oxalic acid without using an excess. Filter, extract the precipitate with boiling water, unite the filtrates, and evaporate until it becomes a thin syrup. The last of the evaporation should be performed on a water bath, to avoid burning. Allow it to stand and crystallize. Tyrosine at first crystallizes out, mixed with a little leucine. Filter, concentrate the filtrate, and allow the leucine to crystallize.

Another better way to separate the mixed substances is to dissolve them in a large quantity of boiling water to which ammonia has been added. To the boiling solution add basic lead acetate solution until the precipitate formed is nearly white. Filter, heat the filtrate to boiling, neutralize with sulphuric acid, and filter while hot. After cooling, the tyrosine crystallizes and can be filtered off, while the leucine remains in solution. Remove the lead from the filtrate which contains the tyrosine by passing hydrogen sulphide through it, filter, and concentrate the filtrate, then boil it with freshly-prepared copper hydroxide. (This is made by precipitating a solution of copper sulphate with sodium hydroxide and washing by decantation until it no longer has an alkaline reaction.) Part of the leucine is thrown down as the copper salt and a part remains in solution. The precipitate can

be removed by filtration and the leucine obtained in the pure state by removing the copper by hydrogen sulphide and allowing the leucine to crystallize after concentration. From the deep blue filtrate the copper salt can be obtained by evaporating it to a small volume and letting it crystallize. It forms aggregations of sky blue crystals. If desired, the copper can be removed from these also by hydrogen sulphide. The leucine thus obtained is not as pure as the first portion.

319. Test the tyrosine with Millon's reagent. It gives a red color, showing the presence in the molecule of the group— C_6H_4OH . (Hoffman's test.)

320. To a portion of tyrosine crystals as large as half a pea add, in a porcelain dish, a few drops of concentrated sulphuric acid and warm on the water bath. This forms tyrosine sulphuric acid. This is diluted with water and enough barium carbonate added to neutralize it, then filtered. The filtrate contains the tyrosine compound, which, with a very dilute solution of ferric chloride, gives a deep violet solution of tyrosine ferric sulphate. (Piria's test.)

321. Warm the tyrosine with a mixture of formalin, 1 part; water, 45 parts; concentrated sulphuric acid, 55 parts. An emerald green color results. (Mörner's test.)

322. Evaporate a small portion of the leucine crystals with a drop of nitric acid upon platinum foil. The residue is colorless, but on adding a drop of sodium hydroxide becomes yellow to brown, and on gently heating rolls around on the foil in the form of drops. (Scherer's test.)

323. Place a few crystals of leucine in a dry test tube and heat gently. They melt with the odor of aminylamine and sublime, the leucine appearing on the sides of the tube as wooly flakes. This will not succeed if the leucine is very impure.

In the mucous cells of the small intestine are formed a number of enzymes; enterokinase which activates trypsinogen; erepsin, which acts upon the secondary derived proteins—the peptones, etc.,—converting them into

amino acids; a nuclein-splitting enzyme, and several which hydrolyze the disaccharids.

324. Demonstrate the action of enterokinase by taking pancreas and section of duodenum from a recently killed animal. Grind with sand 5 to 10 grams of each in separate mortars, with the gradual addition of ten times as much water. Filter them through moistened muslin. Place 10 c.c. of the pancreatic extract in each of three test tubes and make them slightly alkaline with the same amount of sodium carbonate solution.

To *A* add 5 c.c. of water; to *B*, 5 c.c. of unboiled duodenal extract; to *C*, 5 c.c. of boiled duodenal extract. Place in each a shred of fibrin of the same size; not larger than a pea. Mix and let them stand at 38°. Explain the digestion in *B* and its absence in the other tubes.

325. Prepare erepsin by scraping off the mucous surface with a piece of sharp glass or knife; cover the shredded membrane with glycerol, or water and toluol, and let it stand for a day or two, shaking occasionally.

326. Take 10 c.c. of an 8 per cent. solution of Witte's peptone in each of two test tubes and make them slightly and equally alkaline with sodium carbonate. To one add 2 to 3 c.c. of boiled erepsin solution, to the other of unboiled solution. Mix with a drop of toluol and keep the tubes at about body temperature for a day or two, then boil and filter. Try equal volumes with the biuret test, avoiding an excess of the copper sulphate.

The tube with the boiled extract should become pink from the peptones. The one with the unboiled extract should show very little or no pink as most of the amino acid decomposition products of the peptones do not give the biuret reaction. An excess of the copper solution may give a blue color and should be avoided.

What function of the erepsin is shown?

327. Show the presence of the inverting ferment, sucrase, in the intestinal extract by testing a 1 per cent cane sugar solution. (This must be free from dextrose, *i.e.*, have no reducing action.) To 10 c.c. of the sugar solution add 2 to 3 c.c. of the extract with toluol as before and keep at 38° for several hours. As a

control solution use a similar tube with boiled extract instead of the unboiled. At intervals, after two hours, test portions with Trommer's, or some other test, for reducing sugars. In the unboiled tube the sucrose has been inverted and the dextrose reduces.

THE BLOOD.

In the examination of the blood it is convenient to consider it as composed of two parts: the organized elements, like the corpuscles, and the albuminous liquid in which they are suspended,—the plasma. The plasma, on standing, separates into two parts by coagulation, the clot—or fibrin—and a liquid,—the serum.

The reaction of the blood is alkaline to litmus from the presence of the carbonate and phosphate of sodium. The specific gravity varies from 1.045 to 1.075, with an average for adult human beings of about 1.055.

The color of the blood is caused by the red corpuscles (erythrocytes). Even comparatively thin layers of the blood are opaque from their presence. The coloring matter (hemoglobin) can be set free from the corpuscle by water or by many chemical reagents. The color becomes then much darker, since the light is no longer reflected from the surface of the corpuscles. This process is called *laking*, or hemolysis. The addition of strong neutral salt solutions to blood turns it bright red, because of the increased reflection of light from the shriveled corpuscles.

The red corpuscles of the same species of animals have the same shape. The average size of those of one animal of a species will be the same as that of any other, although the size of the individual corpuscle may vary greatly in the same animal. In most mammals they are round, biconcave, non-nucleated disks. In the blood of

birds, amphibians, and most fishes they are nucleated and more or less elliptical. A single corpuscle when seen under the microscope has a yellowish color, not a red. The size of the red corpuscles can be greatly changed by adding water or strong solutions of neutral salts. When water is added it passes in by diffusion, and the corpuscle swells and may burst. Likewise by diffusion when they are placed in a liquid which contains more salts than the blood water passes out and the corpuscle becomes smaller and shriveled in appearance. In order to dilute the blood without changing the size a solution containing 0.9 per cent. of sodium chloride can be used. Such a solution is called *isotonic*; that is, its osmotic pressure is the same as that of the fluid within the corpuscles.

Freshly drawn blood, when allowed to stand undisturbed, in a few minutes becomes thickened to a dark red gelatinous mass. This is due to the coagulation of the colloidal protein, fibrinogen, to an insoluble modification called fibrin. If the coagulation is slow the red corpuscles have time to sink and collect with the fibrin in a mass in the lower part of the vessel. The serum is squeezed out of the mass and surrounds it, above and at the sides. If the blood is beaten during the time of coagulation the fibrin does not separate as a gelatinous substance, but in stringy masses, which have a high degree of elasticity. The coagulation can be prevented or hindered by cold and by the addition of neutral salts, peptones, and some other substances.

To determine the number of red corpuscles the apparatus of Thoma-Zeiss may be employed. This consists of two pieces: a pipette for measuring and diluting the blood and a cell for counting the number with the aid of a microscope. The lower part of the pipette is a graduated capillary tube for measuring the blood. Above is a bulb which, being filled to the mark with

the diluting fluid, dilutes 200 times the blood which was measured in the capillary tube. The counting-cell when covered with a cover glass gives a layer of blood 0.1 mm. in depth. On the bottom of the cell are ruled sixteen squares, each $\frac{1}{400}$ of a square mm. in area. They are surrounded by two rows of smaller rectangles. The volume of blood over each of these squares, then, must contain $\frac{1}{4000}$ c.mm. If the number of corpuscles which are contained in this $\frac{1}{4000}$ c.mm. is determined, the number in any volume can be found by multiplication. Several different solutions have been proposed for the dilution of the blood, one of the most convenient being a 3 per cent. solution of sodium chloride. For clinical purposes the blood is best obtained from the tip of the finger or the lobe of the ear. For testing the method defibrinated blood from the slaughter house may be employed. The dilution may be made twice as great by filling the capillary only to the 0.5 mark and diluting as before. In this case the number of corpuscles in each square must be multiplied by 8000 to give the number in a c.mm.

The average number of red corpuscles normally present is 5,000,000 per c.mm. in the case of a man, and 4,500,000 per c.mm. of a woman. This may vary greatly in disease.

328. Determination of the Specific Gravity of Blood.
—Prepare a mixture of benzene and chloroform, of which the specific gravity when tested with a sensitive hydrometer shall be somewhat less than that of blood. Into this mixture allow a drop of blood to fall. Freshly drawn blood—for example, from the end of the finger—is best, though the method can be demonstrated by the use of defibrinated blood. When the blood has sunk, add chloroform, drop by drop, stirring meanwhile, until the drop floats in the midst of the liquid; that is, it has the same specific gravity. Then filter out the blood, covering the funnel to prevent evaporation, and determine the specific gravity of the mixture by means of a sensitive hydrometer. The mixed liquids can be preserved for future tests.

329. Wind a string around one of the fingers until it is congested, then prick it at the root of the nail with a needle or knife, sterilized by passing through a flame. Test the reaction of the blood, using a piece of neutral, glazed litmus paper. After rinsing off the blood with a little distilled water the paper is blue.

330. Will it react alkaline to all indicators, for example, with phenolphthalein paper? How can a liquid be alkaline to one indicator and neutral to another?

THE RED CORPUSCLES.

331. To 5 c.c. of defibrinated blood which has been shaken with air in a test tube add half its volume of 10 per cent. sodium chloride solution. In another tube do the same, using 0.9 per cent. salt solution. In a third tube repeat, substituting water for the salt solution. Is there any change in color? Put a drop of each mixture, also one of the untreated blood, on a slide and examine under a microscope. Compare size and shape of corpuscles. Relate your observation to change in color.

332. Show that the blood can be laked by adding a few drops of (a) ether, (b) chloroform, (c) solutions of bile salts, (d) by freezing and thawing, (e) by heating to 65° . Explain the nature of the change.

333. **Determination of the Number of Red Blood-corpuscles.** —Fill the capillary tube of the pipette with blood to the mark 1, drawing it in slowly by suction with the mouth and avoiding the presence of air-bubbles in the tube. Quickly wipe dry the end of the pipette with filter paper and draw in the diluting salt solution (3 per cent.) to the mark 101. Close the lower end of the pipette with the finger, then compress the rubber tube at the upper end of the pipette and shake to thoroughly mix the fluids. The small glass bead in the bulb aids in this mixing. Allow the salt solution to flow out of the capillary, place a drop of the

diluted blood on the ruled side and cover it with a cover glass so that air-bubbles are not inclosed. When the corpuscles have come to rest, count the number in all sixteen squares. Count those upon the upper and left hand line of each square as belonging to the square. Use an objective which will magnify 100 to 200 diameters. The squares should be taken in some definite order to avoid counting the corpuscles in the same one more than once. The average number of corpuscles multiplied by 4000 gives the number in a c.mm. of the diluted blood. Multiply this by 100 for the original blood. Since a slight error in the average of each square would make a considerable error when multiplied by 4000, it is advisable to repeat the filling of the cell several times before taking the average. After using, the pipette should be rinsed out first with the diluting salt solution, then successively with water, alcohol, and ether, and finally dried by blowing dry air through it.

334. Separation of the Corpuscles from the Serum.

—Add 30 c.c. of a saturated solution of sodium chloride to 270 c.c. of water, then mix with it 30 c.c. of blood, which has been defibrinated by beating it while freshly drawn. Pour it into a flat-bottomed, shallow dish and allow it to stand until the corpuscles have settled. If a centrifuge is available its use will much shorten the time required. Decant the serum, and, after mixing the corpuscles with more salt solution as before, allow to settle and decant again. By this means the serum can be entirely removed. Preserve the first portion of the solution for testing in 339 and 340. If any of the corpuscles have been hemolyzed it may be reddened.

COAGULATION.

335. Place in a test tube 5 to 10 c.c. of a cold, saturated solution of sodium sulphate. Open the carotid of a rabbit or other small animal and allow twice as much blood to flow into the tube. Collect as much more in a clean, perfectly dry tube and allow both to stand twenty-four hours. In the first tube there is

no coagulation, but the corpuscles settle toward the bottom. In the second the corpuscles are mostly held in the mass of coagulated fibrin from which drops of serum are pressed out.

336. Place in a beaker or flask 3 to 4 c.c. of a 4 per cent. solution of neutral potassium oxalate. Insert a cannula into the carotid artery of a rabbit or cat and let 30 to 40 c.c. of blood flow into the solution without first coming into contact with the glass. Let it stand in a cool place or whirl it in a centrifuge until the corpuscles have settled. The clear liquid above is oxalate plasma. Pour it into a test tube and notice that it does not clot. Add a few drops of calcium chloride solution when a clot forms. (Compare this with the action of rennin 281.)

337. From the oxalate plasma precipitate the fibrinogen with an equal volume of saturated salt solution. Apply to it the protein reactions.

Notice that the serum does not clot with calcium chloride. Why not?

What have you proved of the composition of plasma?

THE SERUM.

338. Draw the blood from the animal into a dry vessel and let it stand at a low temperature (but above freezing) until coagulation has taken place. Some of the serum will have been pressed out by the contraction of the coagulated mass. Decant this and use for the tests.

339. Show that the serum contains protein by testing a diluted solution with

- A. Heat.
- B. Biuret test.
- C. Xanthoproteic test.

340. Separate the proteins of the serum by adding to a portion an equal volume of a saturated solution of am-

monium sulphate. This precipitates the serum globulins. Filter and show that the precipitate contains globulin,—

A. It gives the general reactions of the proteins, such as the xanthoproteic and Millon's.

B. It dissolves in neutral salt solution, as shown by adding a little water to reduce the concentration of the ammonium sulphate, and it reprecipitates from this solution by diluting with many times its volume of water. Why?

To the filtrate from the globulins add enough crystals of ammonium sulphate to completely saturate it. Serum albumin precipitates. Filter it out and show that it gives the xanthoproteic and other protein reactions but does not precipitate upon extreme dilution of its solution. Why not?

341. To about 20 c.c. of blood in a porcelain dish add enough water to hemolyze the corpuscles, acidify slightly with acetic acid, boil and filter. Test the filtrate for sugar, chlorides, phosphates, and sulphates,—

A. Test 5 c.c. by Fehling's or Trommer's test for dextrose.

B. Acidify 5 c.c. with nitric acid and add silver nitrate. A white precipitate or opalescence shows the presence of chlorides.

C. Acidify 5 c.c. with nitric acid, add a little ammonium molybdate solution and warm. Phosphates give a yellow precipitate.

D. Acidify 5 c.c. with hydrochloric acid and add barium chloride. A fine white precipitate indicates sulphates.

342. What have you proved of the composition of the serum? Explain hemolysis from the viewpoint of physical chemistry.

FIBRIN.

343. Prepare fibrin by beating freshly drawn blood with a fork or a bundle of switches. When it has coagulated, pour off the liquid and preserve it for further tests. Wash the fibrin, at first in water to which a little salt has been added, then in clear water. Break up the large clots and continue the washing until the coloring matter is removed. If it is desired to keep it, it can be preserved by drying or in a 1 per cent. solution of mercuric chloride.

344. After noticing the structure and elasticity of fibrin, apply the following tests:—

A. Xanthoproteic.

B. Insolubility in hot and cold water, and in dilute salt solutions.

345. Let a shred of washed fibrin stand for an hour or two in a test tube with 0.1 per cent. HCl. It swells up and becomes transparent, but does not dissolve. Warm at 60° to 70° for several hours; filter and test the filtrate for acid albumin by neutralizing. What is the explanation?

HEMOGLOBIN AND ITS DERIVATIVES.

- I. HEMOGLOBIN: Composed of a simple protein and an iron compound,—hemochromogen.
- II. OXYHEMOGLOBIN: A compound of oxygen with hemoglobin.
- III. METHEMOGLOBIN: Composition same as oxyhemoglobin. Different arrangement of the atoms.
- IV. HEMATIN: The iron compound united with a simple protein in oxyhemoglobin. Hemochromogen plus oxygen.

- V. HEMIN: A compound of hematin with HCl, one molecule of each.
- VI. HEMOCHROMOGEN: Hemoglobin minus its simple protein constituent. With oxygen it gives hematin.
- VII. HEMATOPORPHYRIN: Formed by the removal of iron from hematin, hemin, etc.

Hemoglobin.

Hemoglobin, sometimes called reduced hemoglobin, is the coloring matter of venous blood. It contains iron, besides the elements which enter into the composition of simple proteins. The constitution of the molecule has not yet been determined, although the formulæ of some varieties are known approximately; but it is very complex, containing a large number of atoms. Like the other members of the conjugated protein class, it contains a simple protein—globin—united in this case with an iron compound. It easily unites with oxygen from the air, taking up one molecule of oxygen for each molecule of hemoglobin and forming the readily decomposable compound, oxyhemoglobin. It also forms compounds with carbon monoxide (CO), nitric oxide (NO), and sulphur, all of which are similar to its oxygen compound.

Hemoglobin can be obtained from oxyhemoglobin by the removal of oxygen. This may be effected either by a vacuum, by driving it out by means of a gas which itself does not act on the blood, or by the use of some chemical reducing agent. It is obtained in the crystalline state with more difficulty than its oxygen compound. It is soluble in water, giving a reddish purple solution.

The spectrum of hemoglobin is of great value in testing for its presence, and the same might be said in the case of the hemoglobin derivatives. When a dilute solution of blood is held before the slit of a spectroscope, the tube being turned toward a window, the solar spectrum, consisting of the seven primary colors crossed by fine dark lines, is seen, and in addition one or more dark bands, which are due to the coloring matters of the blood. That of hemoglobin has one broad band with rather indistinct edges lying between the *D* and *E* lines of the solar spectrum. If the liquid in the tube be shaken with air oxyhemoglobin is formed, which has two dark bands. For clinical purposes the direct vision or pocket spectroscope will probably be found to be the most convenient form of instrument. (Figs. 1 and 2 on Plate IV show the spectra.)

A fresh alcoholic solution of guaiacum when oxidized gives a blue color. Many oxidizing agents, like hydrogen peroxide and oil of turpentine which has absorbed oxygen by standing for some time exposed to the air, will not act on the guaiacum alone, but will do so if hemoglobin or its compounds are present to serve as a carrier of oxygen. This is often used as a test for blood, but is only useful in a negative way, for protoplasm will give the same reaction. It can consequently be obtained from milk, pus, or mucus, or even from such substances as the potato. If the reaction fails, however, there can be no blood present.

Benzidine when oxidized gives a blue compound. As in the case of guaiacum, hydrogen peroxide alone will not react with it, but does so in the presence of blood pigments. It is a very sensitive reaction.

Since hemoglobin contains a simple protein as a part

of its molecule, it will give the general reactions of the protein compounds.

The determination of the amount of hemoglobin in the blood is made by comparing the color of the diluted blood with a solution containing a known weight of hemoglobin, or with some other colored liquid or solid. For making a standard solution of hemoglobin the recrystallized substance is used. A solution of this can be preserved in a corked bottle or sealed tube for an indefinite time. The strength is ascertained by evaporating to dryness a given volume, and weighing the residue of hemoglobin. This method gives accurate results.

346. Determination of the Amount of Hemoglobin in Blood.—Dilute a solution containing a known weight of hemoglobin with distilled water until it is a very light red color. Dilute in the same way the blood to be tested until the color is the same. For comparison of colors the two solutions may be placed in flat-bottomed tubes of colorless glass (Nessler tubes) or in a colorimeter. Reckon from the amount of dilution the amount of hemoglobin compared with the standard solution, also the absolute weight and percentage.

For clinical purposes a convenient instrument for the determination of the amount of hemoglobin in the blood is that of *Fleischl*, called an hemometer. It consists of a short, vertical cylinder for holding the blood, separated by a partition into two compartments; a long movable wedge of ruby glass under one compartment for a standard of color, and a white surface below for reflecting the light up through the wedge and cylinder to the eye of the observer. The amount of hemoglobin is found by filling one compartment of the cylinder with diluted blood, and the other, over the ruby prism, with water. The prism is then moved until the depth of color is exactly the same as that of the blood, when the percentage of hemoglobin compared with

the normal amount can be read from the scale. The following is the process:—

Use for a light a lamp or yellow gas flame, not an incandescent light or daylight. Any blood may be used for practice. In clinical cases use that obtained from the tip of the finger by the aid of a lancet. After making the incision force out a drop of blood by gentle pressure. Measure off the required volume of blood ($6\frac{1}{2}$ c.mm.) by filling the small glass tube, open at both ends and mounted on a handle, which accompanies the hemometer. This is accomplished by holding it horizontally and dipping one end into the drop. Wipe carefully all blood from the surface of the tube. This will be more readily done if the surface of the tube is kept slightly greasy by being preserved in an oily piece of chamois. The blood must exactly fill the tube, having neither a convex nor a concave surface at the open ends.

The compartment over the ruby glass prism is to be filled with distilled water by means of a pipette and the other one not more than a quarter full. Into the latter the open glass measuring tube is dipped before the blood has coagulated, and the hemoglobin is dissolved by moving the tube back and forth so as to wash out the blood. Then rinse off the tube into the blood solution by the use of a few drops of water from the pipette. Fill the compartment from a half to three-fourths full of water and stir well with a wire or with the handle of the measuring tube. Mix the water with the blood until no turbidity is seen and until it is evident that the fluid in the angles is completely incorporated with the rest. Now drop water from the pipette upon the blood solution until it, as well as the water in the other compartment, comes exactly to the top of the division between the compartments. If the tip of the pipette is placed slightly below the surface and the water flows slowly it will not mix with the solution of blood below. This is advisable in order to prevent the possibility of any of the hemoglobin's passing over into the other compartment. If both compartments are filled to the top of the separating partition, so that there is no meniscus at the top of the liquid, they appear, when looked at from above, to be separated only by a narrow black line. A little grease on the top of the partition will help to prevent a mixing of the two liquids. (Some authors recom-

mend that the water be allowed to rise above the partition and a cover glass be then laid on top to prevent currents.)

Place the instrument with the large screw at the right, turn the reflector so as to illuminate the solution, shade the eye from other light, and move the ruby prism so that the shades of red in the two compartments are the same. The figure in the scale, opposite the middle of the cylinder, gives the percentage of hemoglobin as compared with the average amount found in normal human blood.

With Dare's hemoglobinometer but a single drop of blood is necessary. This is drawn by pricking the skin with a needle and allowed to run between two parallel plates of glass fixed at a short distance apart. Comparison is made with the color on the margin of a disk which can be rotated by means of a milled screw until the same shade is attained. Yellow light is transmitted through the blood and colored glass from a candle held opposite; the percentage of normal hemoglobin is read from a scale on the edge of the revolving disk. The operation is simpler and more expeditious than that of Fleischl.

Oxyhemoglobin.

The crystalline form of oxyhemoglobin differs when its source is from different species of animal: from human blood being in long prisms; from the squirrel, flat, six-sided plates; and from the guinea pig, tetrahedra. It can be easily crystallized from the blood of the dog, guinea pig, and rat, but with more difficulty from human blood or ox blood. The color of the crystals is a bright red, and their solution is a much brighter red than that of hemoglobin, which, when pure, approaches a black.

Oxyhemoglobin is formed by the union of a molecule of oxygen with one of hemoglobin, and it can without difficulty be changed back into hemoglobin. The molecule of oxygen is in this compound very loosely united. Oxyhemoglobin may be also considered as composed of an iron hematin, with a simple protein. It is decom-

posed into these two substances when its solution is heated, this change being hastened by acids or alkalies. When heated with glacial acetic acid and a little sodium chloride it is decomposed, the hematin uniting with the nascent HCl formed at the same time and giving hemin, the microscopic crystals of which have a brown color and characteristic form. This is one of the best proofs for the presence of blood, although it does not distinguish between the different kinds. No other known substance gives crystals of this color and shape.

The spectrum of oxyhemoglobin consists of two dark bands: a narrow one at the right of the *D* line in the yellow and a broader and less distinct one in the green at the left of the *E* line of the solar spectrum. They can be made to vary in width as well as distinctness by making the solution more or less dilute. They can be perceived when it contains 1 gram of oxyhemoglobin in 10 liters of water; that is, 1 part in 10,000. If to this solution is added a few drops of ammonium sulphide, which has a reducing action, the oxygen is removed and in a few minutes the one broad band of hemoglobin is seen in that part of the spectrum between the two oxyhemoglobin bands. It is not so distinct as are the two bands and the solution may have to be strengthened to make it plainly visible. The two bands reappear upon shaking the solution with air, the oxyhemoglobin being formed again.

Methemoglobin.

In its percentage composition methemoglobin differs very little, if any, from oxyhemoglobin, and probably is formed by a rearrangement of the atoms in the oxyhemoglobin molecule. It is produced whenever oxy-

hemoglobin is dried in the air at ordinary temperatures or when it is acted upon by weak acids. Certain oxidizing agents also will convert oxyhemoglobin into methemoglobin. It is also found in transudations and cystic fluids which contain blood; moreover in the urine during hematuria and hemoglobinuria, as well as in the blood itself in certain cases of poisoning or after a large destruction of blood corpuscles by burns of the skin.

In the methemoglobin molecule the oxygen is more firmly attached than in oxyhemoglobin, being removable neither by a vacuum nor by another gas. Like oxyhemoglobin, however, it is changed by weak acids or alkalies into hematin and a simple protein. Like oxyhemoglobin, too, it is converted by reducing agents or by putrefaction, where reducing forces are at work back into hemoglobin.

Methemoglobin crystallizes in brownish red needles or sometimes in plates. It is easily soluble in water, giving a brown solution, which becomes red on the addition of an alkali. The spectrum of the alkaline methemoglobin solution has two bands much similar to those of oxyhemoglobin, one on the *D* line, the other near the *E* line.

Hematin and Hemin.

Hematin is the iron compound which is combined with a simple protein to form oxyhemoglobin. It is set free whenever oxyhemoglobin is decomposed by the action of the gastric or pancreatic juice or by an acid. It is consequently found in the intestine after the eating of meat; also in the stomach after poisoning by a mineral acid.

The formula is given as:—



It may be obtained from hemin, its compound with hydrochloric acid. It is an amorphous substance, dark brown or bluish black.

Hemin is formed by the action of hydrochloric acid on hematin, either directly or by first decomposing the oxyhemoglobin. It forms microscopic crystals which, in a large amount, have a blue-black color. Under the microscope they are brown, rhombic prisms. They are sometimes separate, but two are often crossed or several are collected in clusters or rosettes. (Plate I, 3.) They are insoluble in water, but dissolve in alkalies. They are often called Teichmann's crystals, and are important in proving the presence of blood.

Carbonic Oxide Hemoglobin.

When carbonic oxide, either pure or mixed with other gases, is breathed or passed through blood, it unites with the hemoglobin, forming CO-hemoglobin: a compound similar to oxyhemoglobin. It is, however, a more stable compound, the oxygen being unable to drive out the CO and take its place. Consequently the hemoglobin is rendered useless as a carrier of oxygen, and cases of poisoning by coal gas, which contains carbon monoxide, are often followed by fatal results. The compound has a two-band spectrum much like that of oxyhemoglobin, but it differs in not being changed to hemoglobin by reducing agents. The crystals are similar to those of oxyhemoglobin, but more of a bluish red. When mixed with strong sodium hydroxide solution blood containing carbonic oxide gives a red mass, while pure blood turns brown, with a greenish cast.

Hemochromogen.

As the oxyhemoglobin by the action of acids or alkalies is decomposed into a simple protein and hematin, so by the same agencies hemoglobin gives a simple protein and hemochromogen. The latter in the presence of free oxygen is converted into hematin. Hence, as we should expect, by the removal of oxygen from hematin by the aid of reducing agents we obtain hemochromogen. The spectrum of hemochromogen, in an alkaline solution, has two bands similar to those of oxyhemoglobin, but a little farther toward the blue. The color of the alkaline solution is a cherry red. It is often seen in specimens of the liver, muscles, etc., which have stood for a time in alcohol.

Hematoporphyrin.

By the action of acids upon hematin the iron is removed, leaving a violet to red coloring matter—hematoporphyrin. It is found in the contents of the stomach and intestine after poisoning with strong acids. It also is found in some of the dark colored urines. It is insoluble in water, more soluble in acids, and easily so in alkalies.

347. Dissolve a little dried blood in nitric acid with the aid of heat. Filter and test the filtrate for iron with a few drops of potassium ferrocyanide, which produces a blue color. If an excess of ferrocyanide is used the color may be green. Why?

348. Add enough blood to a little water to color it a bright red. Dissolve a small crystal of ferrous sulphate and as much tartaric acid in 10 c.c of water, then enough ammonia to make it faintly alkaline (Stokes' reagent). Add one drop to the blood solution. The oxyhemoglobin gives up its oxygen to the iron compound, becoming changed in a short time to hemoglobin, as is shown by the dark color. An excess of the reducing solution should be avoided.

349. Shake the dark solution of hemoglobin with air and notice the change in color to a scarlet, showing the formation of oxyhemoglobin.

350. A description of the spectroscope can be found in any standard work upon physics. Examine a very dilute solution of blood with the spectroscope in the following manner: First examine the solar spectrum by looking through the spectroscope with its slit directed toward a window. Close the slit to a very narrow opening, and focus by sliding the focusing tube until the fine, dark lines are seen clearly. There are hundreds of these so-called Fraunhofer lines in the spectrum of the sun, but with an ordinary instrument many are indistinct. A few of the most prominent should be noticed and used to locate the position of the dark bands in the spectra of hemoglobin and its derivatives. The most noticeable are the *C* line in the red, the *D* line in the yellow, the *E* and *b* not far apart in the green and *F* in the blue. If there are fine black lines running lengthwise of the spectrum they are caused by the dust in the slit. Next make solutions of blood and water of different dilutions and examine the spectrum which is given when a test tubeful of the solution is held before the slit after the solution has been shaken with air to form oxyhemoglobin. Notice the position of the two bands and observe that this does not change with the different dilutions, although the bands may be wider or more distinct when the solution is concentrated.

351. To the solutions of oxyhemoglobin add a few drops of ammonium sulphide, and after they have stood a short time examine them again with the spectroscope. Notice that when the oxygen has been removed by the

ammonium sulphide the spectrum has changed to that of hemoglobin, which consists of one broad band in the space between that formerly occupied by the two. It is less distinct than the two bands of the oxyhemoglobin spectrum.

352. Shake the reduced solutions with air and see that the two bands have returned. If any ammonium sulphide remains the oxyhemoglobin will be changed again to hemoglobin on standing.

353. Through the solution of blood pass a stream of illuminating gas for a few minutes. The carbon monoxide is absorbed, forming carbonic oxide hemoglobin. Examine it with the spectroscope. It gives two dark bands much like those of oxyhemoglobin, though the one next the green is not as wide as in the oxyhemoglobin spectrum. Try now to reduce the compound to hemoglobin by means of ammonium sulphide. The carbonic oxide is not expelled, the two bands remaining unchanged.

354. Prepare an alcoholic solution of guaiacum by dissolving some of the gum taken from the middle of a lump. No blue color is produced in the solution on the addition of a small amount of old oil of turpentine or hydrogen peroxide, but it is produced upon the further addition of a few drops of blood. Explain the reaction.

355. Try the same test on the scrapings from a potato. A blue color is produced on standing, without the aid of the turpentine or any similar oxidizing agent. Explain how.

356. Add to dilute blood an acetic acid solution of benzidine, then a few drops of a dilute solution of hydrogen peroxide. A

blue color results. Try extremely dilute solutions of blood in order to form a judgment as to the sensitiveness of the reaction.

357. To 2 to 3 c.c. of defibrinated blood add as much 2 per cent. hydrogen peroxide. Relate the result to the guaiacum and benzidine reactions.

358. Reduce fluorescein by warming 10 c.c. of a solution (0.1 gram in 100 c.c. of 10 per cent. sodium hydroxide) with about $\frac{1}{2}$ gram of powdered zinc until it is colorless. Make dilutions of blood in the proportion of 1 drop in a liter, 1 in 10 liters and 1 in 100 liters. Treat about 10 c.c. of each of these in test tubes with 2 to 3 c.c. of the reduced fluorescein solution and compare the results with the action of the reduced fluorescein solution on 10 c.c. of distilled water, letting them all stand ten minutes. In the presence of oxygen carriers like the blood pigments the liquid becomes a fluorescent green color. Explain all changes.

359. Acidify slightly a solution of blood, and heat to boiling. Notice the coagulated albumin and the dark colored hematin coming from the decomposition of the oxyhemoglobin.

360. Filter out the hematin, or use instead a drop of fresh blood, and prepare hemin crystals from it by first drying thoroughly on a glass slide, then adding a minute amount of sodium chloride. Cover with a cover glass; add a drop of glacial acetic acid, which will flow under the cover. Then heat over a small flame until the acid boils. After cooling, examine under a microscope. The crystals usually are better if the acid is added and the slide heated two or three times. Compare with Plate I, 3.

361. Explain the action of the acid on the salt, of heat upon the blood pigment, of the two products upon one another. Give composition of the crystals.

362. To prepare a large amount of hemin, precipitate the corpuscles from defibrinated blood by the addition of a large excess of a salt solution which contains 1 volume of a saturated salt

solution in 10 to 20 volumes of water. After twenty-four hours pour off the solution and rinse the precipitated corpuscles into a flask by the aid of a small amount of water. Add half its volume of ether, shake, and after pouring off the ether which removes most of the fats allow the solution of blood coloring matters to evaporate in flat dishes at ordinary temperature to a syrup. Mix this with 10 to 20 volumes of glacial acetic acid and heat in a flask one or two hours on the water bath. Then pour into a beaker, add several volumes of water, and allow to stand several days. Wash with water and remove the albuminous substances by boiling with acetic acid. Explain all changes.

363. Saturate 2 or 3 c.c. of blood with carbonic oxide by passing illuminating gas through it. Add to it twice its volume of sodium hydroxide solution,—specific gravity, 1.3,—containing about 27 per cent. NaOH. Do the same with pure blood, and spread the products on a piece of porcelain. Notice that the pure blood gives a brown color, with a shade of green. The carbon monoxide blood has a bright red color on porcelain. This is a useful test in cases of suspected poisoning by carbon monoxide.

364. What property of the carbon monoxide hemoglobin is shown in this experiment? Explain action of the alkali on normal blood.

365. Prepare crystals of oxyhemoglobin by placing on a microscope slide a drop of blood (one which crystallizes easily, like that of a dog, rat, or guinea pig) and cover it with a drop of Canada balsam. Cover the whole with a cover glass and examine it under the microscope. The crystals will form in a few minutes. They can also be made by mixing the drop of blood with a small drop of water on the slide and allowing it to evaporate until a dry ring has formed around it. Place a cover glass over it and it will crystallize. It is well to examine several different species of blood if they can be obtained, such as guinea pig, which gives crystals in the form of tetrahedra (four-

sided); mouse, giving six-sided plates; cat or dog, giving four-sided needles.

366. A large quantity of crystallized oxyhemoglobin can be prepared by the following method: Make a solution of salt containing 1 volume of saturated salt solution to 9 volumes of water: Add 10 volumes of this to 1 of defibrinated blood, and let it stand a day or two in shallow, flat bottomed vessels until the corpuscles have settled. Pour off the clear liquid, rinse the corpuscles into a separatory funnel with the aid of as small a quantity of water as possible, and add about as much ether. Shake, but not too violently, separate the solution of oxyhemoglobin from the ether, and filter the former. Cool it to 0° and mix it with one-fourth its volume of alcohol which has been also cooled to 0° . Let the mixture stand at a temperature of -2° to -10° for several days, until the oxyhemoglobin has crystallized. This occurs with the blood of dogs and rats almost immediately, but that of the ox crystallizes with much more difficulty. After crystallization filter off the crystals in the cold, and dry by pressing between filter paper. The crystals may be purified by dissolving in a small amount of water, cooling and precipitating in the same manner, repeating several times. The crystals can be preserved for a standard in the determination of the quantity of oxyhemoglobin in blood.

367. Test a parchment dialyzer or tubing to see that it has no holes, then introduce defibrinated blood diluted with an equal volume of water. Let it stand an hour or more in water. Notice that the hemoglobin molecules cannot pass out, but the molecules of chlorides do so, as is shown by adding silver nitrate to the outer liquid. What does this prove about the size of the hemoglobin molecules?

368. To a solution of blood in water add a small crystal of potassium ferricyanide. Methemoglobin is formed, the color of the solution changing from a red to a brown. Make it slightly alkaline and it becomes red. Examine the spectrum of the alkaline solution. It is much like that of oxyhemoglobin, though the first band is broader and extends somewhat farther toward the

red. It is reduced to hemoglobin by ammonium sulphide, as is the oxyhemoglobin.

369. Large amounts of crystalline methemoglobin can be obtained by adding to a concentrated solution of oxyhemoglobin enough of a concentrated solution of potassium ferricyanide to give it a deep brown color. Crystallize, as in the case of oxyhemoglobin, by cooling to zero and adding one-fourth the volume of cold alcohol.

370. Hemolyze 1 to 2 c.c. of blood by dropping in water, then add concentrated hydrochloric acid, at first drop by drop, to precipitate the hematin with the coagulated proteins, then enough to dissolve the hematin as acid hematin. Filter if the solution is not clear and examine the spectrum.

371. Prepare alkali hematin by warming nearly to boiling 1 to 2 c.c. of blood with sodium hydroxide solution. Examine the spectrum.

372. Prepare reduced alkali hematin (hemochromogen) by treating a solution of alkali hematin with a little ammonium sulphide or Stokes's reagent and letting it stand a few minutes. Examine the spectrum: it has two bands farther toward the right than that of oxyhemoglobin. This is a very sensitive test for blood, even more so than the oxyhemoglobin spectrum.

373. Form hematoporphyrin by mixing 1 drop of defibrinated blood with 3 to 4 c.c. of concentrated sulphuric acid. The liquid is purple. Examine the spectrum, diluting with concentrated sulphuric acid if necessary. What is the action of the acid on the hematin part of the oxyhemoglobin molecule?

TESTING SUSPECTED STAINS FOR BLOOD.

The following tests can be used, first on a stain made by drying a drop of blood on a piece of cloth, then upon unknown stains.

374. Soak a small portion of the stain in a few drops of water on a microscopic slide. If no color is imparted

to the liquid, blood is not present or the hemoglobin has been so much decomposed that only the guaiacum or hemin test will show its presence, or, possibly, the albumin and globulin have been coagulated.

375. To a few drops of the liquid add as much fresh tincture of guaiacum and a little old oil of turpentine or hydrogen peroxide. If no blue color appears immediately blood is absent or it has been to a great degree, decomposed. The benzidine reaction can be also used here.

376. If a red color was seen in the water try the spectroscopic test. By drying, both hemoglobin and oxyhemoglobin may be changed into methemoglobin, which gives a somewhat different spectrum. (Plate IV, 11.)

Ammonium sulphide reduces this to hemoglobin, and shaking with air then gives the spectrum of oxyhemoglobin (350, 351). If these are all obtained the presence of blood is proved.

377. To confirm the results, or, if the stain is too small to obtain them, try to obtain the hemin crystals as in 360. Apply the test directly to the stain, not to a solution of the stain. After applying the acid to the dried mass the latter should be broken up with a glass rod to insure thorough mixture. If it is very hard let it soak in the acid for a short time. These hemin crystals are produced only from the coloring matters of the blood. When in doubt as to their identity they should be compared with those obtained from known blood, remembering that different specimens of crystals may differ considerably in size.

378. If there is a sufficient amount of the blood, not too long exposed to the air, or if it is desirable to deter-

mine the species of animal, it may be soaked in a small amount of 0.7 per cent. salt solution and the appearance and size of the corpuscles compared with those of known specimens or measured by a microscope with a micrometer eye-piece.

379. Obtain from the instructor several stains and apply the guaiacum, spectroscopic, and hemin tests, also the test for corpuscles. Report positive and negative results according to the following form, and if all tests on the same sample are not positive or negative, explain the discrepancies.

No. of Sample	Corpuscles	Guaiacum	Spectroscopic	Hemin
1				
2				
3				

OCCULT BLOOD.

"Occult blood" is present in the alimentary tract as a consequence of slight hemorrhages or other lesions, in such small amounts that it is not visible. If it is to be tested for, the subject must be kept on a meat-free diet several days, since meat foods will give the reactions.

380. Smear a little of the solid stool on a microscope slide and drop on the benzidine-hydrogen peroxide reagent (356).

381. Or stir a little of the fecal residue with 5 to 10 c.c. of water to a thin suspension and boil. Cool, let it settle and float on top a mixture of hydrogen peroxide with either benzidine or alcoholic guaiacum solution (354).

Blood should give a blue color with either test.

BLOOD TESTS USED IN THE STUDY OF METABOLISM.

Blood for testing can be obtained from the slaughter house, from experimental animals, either by killing or without seriously injuring them, or from human subjects. Anesthetics can be used if desired. Naturally tests should be made as soon as possible.

If the blood is obtained from the slaughter house it should be caught in vessels which are not only clean but also dry. It can be defibrinated by immediate whipping or coagulation can be prevented by keeping it at a low temperature, but above freezing. Addition of soluble oxalates or citrates or of leech extract (hirudin) also prevents clotting.

Small amounts of blood from rabbits or dogs can be drawn from the ear by making a sharp cut in the edge with dissecting scissors, severing some of the small vessels. It should be allowed to drop into a beaker without exerting pressure upon the surrounding tissues. Larger quantities (2 to 5 c.c.) can be drawn by a hypodermic syringe with a sharp needle. This is first rinsed with some concentrated sodium oxalate solution to prevent clotting and the needle inserted into one of the ear veins of a rabbit. Afterward bleeding is stopped by compression a few minutes by artery forceps. Similarly with guinea pigs 2 to 5 c.c. can be drawn through the needle directly from the heart without killing the animal. If larger animals are to be killed they can be anesthetized and a cannula inserted into the carotid or femoral artery.

With human beings small volumes are most conveniently won by puncturing with a lancet the lobe of the ear or the tip of the finger and allowing the blood to flow out without squeezing. Clinically the blood can be drawn from a vein of the forearm. A sharp hypodermic needle is connected by 3 to 4 cm. of rubber tubing to a pipette the inside of which has been moistened by a concentrated oxalate solution then dried by an air current. When the needle has been inserted in the vein it is filled with blood by gentle suction.

Inasmuch as the products of metabolism in the animal body are carried into the blood, quantitative tests may

reveal whether there is a variation from the normal condition. An increase in non-protein nitrogen compounds—urea, uric acid, creatinine, etc.—points to some disease of the kidneys (nephritis) which hinders excretion and thus leads to an accumulation of these substances. In diabetes glucose and the acetone bodies are more abundant. Uric acid is high in gout but not the other nitrogen compounds. Fats and cholesterol may be high with the glucose.

An abnormal oxidation with a formation of excessive amounts of acids and the consequent withdrawal of bases (a condition called acidosis) modifies the blood. It can be produced very quickly in normal persons by excluding carbohydrates from the food.

Acidosis may result in an increased hydrogen ion concentration. This is usually slight, because the acid phosphates and carbonates of the alkalies, e.g., K_2HPO_4 and $NaHCO_3$, react with the hydrogen ion (buffer action) and neutralize it. Substances like the alkaline phosphates and carbonates constitute the *alkali reserve* of the blood; consequently acidosis lowers the alkali reserve, although its presence cannot be detected by such an indicator as litmus.

In consequence of the action of the hydrogen ion upon the acid sodium carbonate,



two results are noticeable,—

the alkali reserve is decreased,
the alkali tolerance is increased.

The alkali reserve can be determined by two methods,—indirectly, by testing the amount of carbon dioxide in

the alveolar air, and directly, by finding the carbon dioxide capacity of the plasma.

When a condition of acidosis brings about a greater decomposition of the bicarbonates of the blood the carbon dioxide thus liberated acts as a respiratory stimulant; respiration becomes more active and the lungs are more thoroughly swept out. Hence the percentage of carbon dioxide in the air of the alveoli is diminished. There is, therefore, a direct relationship between the amount of the carbon dioxide in the alveolar air and the alkali reserve of the blood. Normally in man there is about 5 to 6.5 volume per cent. of carbon dioxide; in acidosis it may drop to 2 or lower.

382. Determination of Alveolar Carbon Dioxide. (Fridericia's method).—One hundred c.c. of air from the lungs are collected in a special apparatus (Fig. 3) and the carbon dioxide absorbed by sodium hydroxide, the decrease in volume representing the carbon dioxide.

While sitting quietly and breathing naturally, the subject fills the tube from the lungs.

Open the stopcocks *a* and *b* into the tube *y*, the passage to *c* being closed. After sitting quietly and breathing naturally the subject fills the tube with alveolar air by breathing quickly into *m* and immediately closing stopcock *a*. The apparatus is placed in a vessel of water at room temperature for five minutes, when the stopcock *b* is turned so as to connect *y* with *c* and shutting in the 100 c.c. of air into the bulb tube. After removal from the water the tube *c* is dipped into 10 per cent. sodium hydroxide solution, which is drawn into tube *y*. Incline *y* slightly downward so as to prevent escape of gas from *x* and force a little of the hydroxide solution into *b* through

x. Close *b* and let the remainder of the hydroxide flow out through *c*. Mix the hydroxide and air for half a minute, when the carbon dioxide has been absorbed. Let the apparatus stand in the water again, which will rise through *c* into *y*. Then open the cock *b* so as to connect *x* and *y*, and let it stand in the water for five

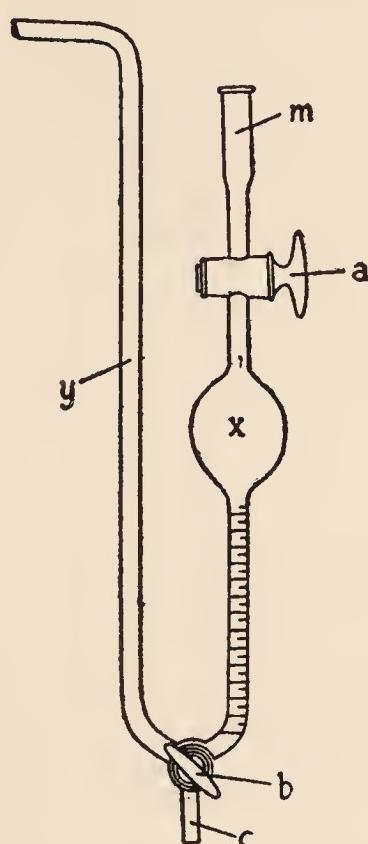


Fig. 3.—Apparatus for determining the carbon dioxide tension in alveolar air.

minutes to equalize the temperature. Bring the water inside and out to the same level by raising or lowering the apparatus and take the reading at the bottom of the meniscus. This represents the volume per cent. of carbon dioxide in the alveolar air.¹

¹ In the Journal of Biological Chemistry, 1918, xxxiii, 277, Van Slyke gives clinical results as to carbon dioxide in alveolar air, normally and in acidosis.

383. The Determination of Carbon Dioxide in Blood Plasma (Van Slyke's method¹).—This is carried out in a special pipette (Fig. 6) in which the carbon dioxide is liberated by acid and shaking in a vacuum, then driven into the measuring tube by a mercury levelling bulb after the liquid has been drawn off. The apparatus can be tested for tightness by filling it with mercury by raising the bulb, closing the cock *e* then lowering

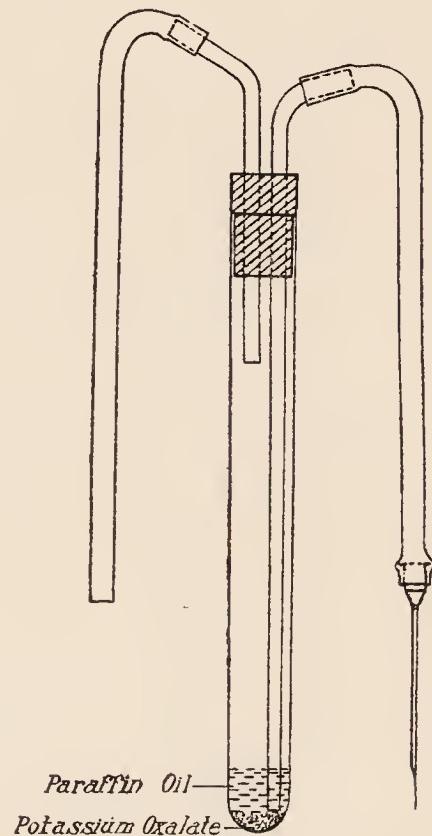


Fig. 4.—Centrifuge tube for collecting blood under paraffin oil without loss of carbon dioxide.

the bulb as far as possible. When it is raised, unless air has leaked in, the mercury strikes the top with a sharp click.

After an hour of rest 6 or 7 c.c. of blood are drawn from an arm vein through a needle into a tube (Fig. 4) containing a little powdered potassium oxalate and paraffin oil, stirred with the inlet tube without shaking, and then centrifuged.

About 3 c.c. of plasma is placed in a separatory funnel connected with a bottle of moist glass beads (Fig. 5). The air in the funnel is displaced by the operator's breathing quickly once

¹ Journal of Biological Chemistry, 1917, xxx, 347.

into it through the beads and closing the stopper just before the expiration is finished. The stopcock is closed and the funnel turned over for two minutes in order to spread the plasma over its surface and complete the saturation with carbon dioxide from the alveolar air of the operator.

Fill the apparatus (Fig. 6, p. 158) including capillaries above upper cock with mercury, wash out cup at top with carbonate-free ammonia and run into the cup 1 c.c. of plasma from a pipette, letting the tip dip below the liquid. With mercury bulb at position 2 and cock *f* as shown in figure admit plasma to pipette leaving enough to fill capillary. Wash twice with 0.5 c.c. of water into bulb, then let 0.5 c.c. of 5 per cent. sulphuric acid

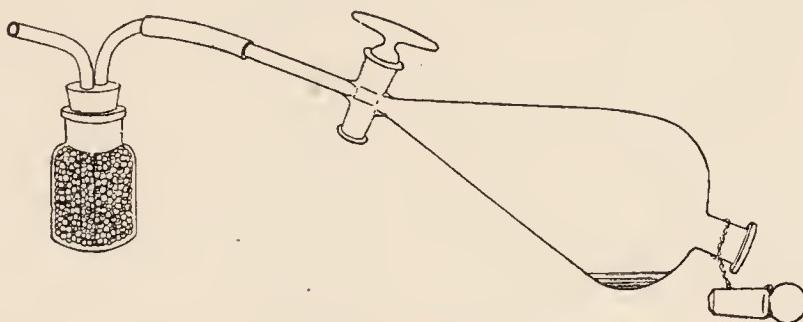


Fig. 5.—Apparatus for saturation of plasma with carbon dioxide from alveolar air.

run in, a total of 2.5 c.c. of liquid. Always leave enough liquid above the cock to prevent access of air. (To prevent foaming of plasma a drop of caprylic alcohol can be admitted before the acid).

Lower the bulb until the mercury has fallen to the 50 c.c. mark thus creating a vacuum, then close the lower cock and turn the pipette upside down fifteen or more times. Open the lower cock so as to allow the liquid to flow into *d* without letting the gas follow it. Then raise the bulb slowly while the lower cock is turned so as to connect the pipette with *c*. Raise the bulb until the mercury is level with that in the tube and the gas is in the graduated tube where its volume is read.

Some air was dissolved in the liquid, varying from 0.951 c.c. at 15° to 0.039 at 30°. Subtract this from the observed volume, calculate the volume at 0° and 760 mm. barometric pressure by the usual methods. If the weight of the carbon dioxide is desired multiply by 1.964 mg., the weight of 1 c.c.

Other indications of acidosis will be discussed under urine. They include alkali tolerance, index of acid excretion, acetone bodies, and excreted ammonium salts.

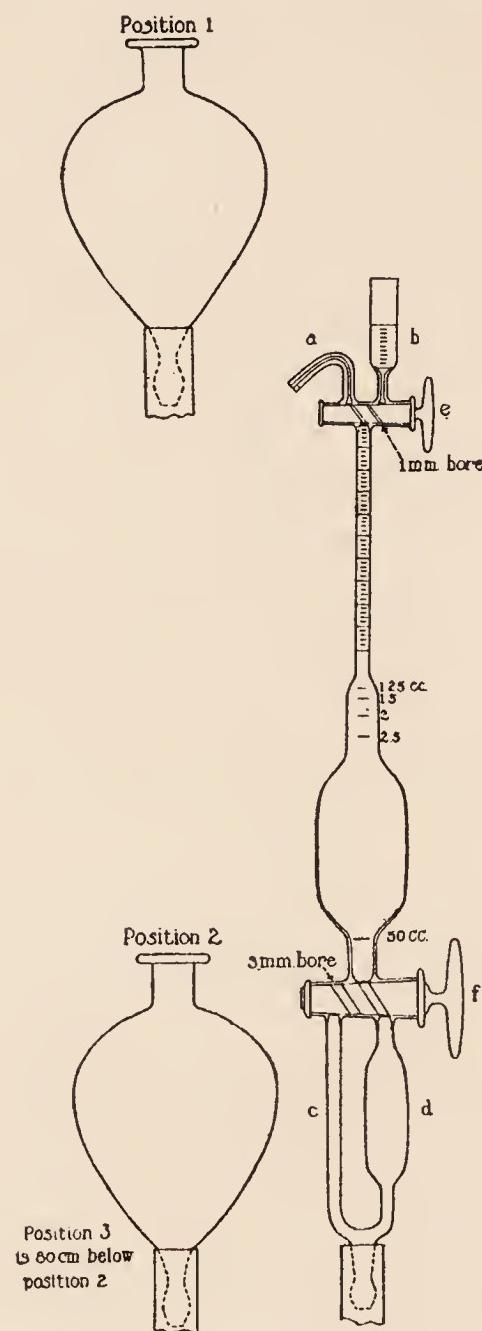


Fig. 6.—Van Slyke apparatus for the determination of carbon dioxide in blood.

384. Determination of the Oxygen Capacity of the Blood (Van Slyke's method¹).—Use the apparatus described for determination of carbon dioxide (Fig. 6).

¹ Journal of Biological Chemistry, 1917, xxxiii, 127.

The blood is first aerated, then introduced into the pipette, the combined oxygen is liberated by potassium ferricyanide, and its volume measured.

Three c.c. or more of blood is spread over the inner wall of a bottle or separatory funnel and rotated a few minutes to ensure aeration, then it is poured into a test tube. The blood apparatus is freed from air by introducing 6 c.c. of ammonia (4 c.c. of the concentrated to a liter of water) and 5 drops of caprylic alcohol. Remove the air by shaking as in CO₂ determination, (383) finally force up about 2 c.c. of air-free ammonia into the cup. Stir the blood thoroughly and, by means of a pipette with two marks, run 2 c.c. under the ammonia in the cup. Let it flow into the pipette washing it in with the ammonia, and a few more drops if necessary. No air should be allowed to enter. After it has completely laked, which may require five minutes, 0.4 c.c. of saturated, air-free potassium ferricyanide solution is introduced. The levelling bulb is lowered until only a few drops of mercury remain above the lower stopcock and the apparatus is shaken, the blood being whirled around over the surface. The oxygen is extracted in about half a minute and can be measured as was the carbon dioxide in the preceding experiment. To make sure the evacuation can be repeated.

The volume can be corrected to 0° and 760 mm. barometer. Subtract from it air dissolved in 2 c.c. of blood, that is from 0.037 c.c. at 15° to 0.028 c.c. at 30°.

A SYSTEM OF BLOOD ANALYSIS.¹

Ten c.c. of blood is sufficient to determine the quantities of non-protein nitrogen, urea, creatinine, creatine, uric acid and sugar. To prevent the coagulation of the blood potassium oxalate can be used, but not more than 20 mg. to 10 c.c. of blood.

385. Removal of Proteins from Blood.—Transfer by a pipette 10 c.c. of blood to a 150 to 200 c.c. flask and mix with 70 c.c. of water. From a pipette add 10 c.c. of 10 per cent. solution of sodium tungstate and mix. From another pipette add to the

¹ From the outline of Folin and Wu. Journal of Biological Chemistry, xxxviii (1919) 81.

flask, while shaking, 10 c.c. of 0.67 normal sulphuric acid; close the flask with a rubber stopper and give a few vigorous shakes; a dark brown coagulum should form; if it does not add normal sulphuric acid drop by drop shaking after each addition and allowing the mixture to stand a few minutes before adding more, until coagulation is complete. Prepare a filter large enough to hold all the liquid, pour it on and cover the funnel with a watch



Fig. 7.—Ostwald pipette for accurate measurement of small volumes of liquid. The stem is a thick walled capillary tube of 1 mm. bore.

glass. Refilter if the filtrate is not clear. It is recommended that the pipettes be cylindrical, with a long tip and graduated from the tip up to 15 c.c. The filtrate should show no acid reaction to Congo red paper.

385a. Determination of Blood Sugar.—The protein-free filtrate is heated with an alkaline copper tartrate solution and the precipitated cuprous oxide is treated with an acidified phosphotungstic-phosphomolybdic acid solution (Folin's phenol reagent). This is reduced with the formation of an intense blue color which is compared with that from a known sugar solution.

The solutions needed are:—

1. A standard sugar solution containing 1 gram of pure anhydrous dextrose in water, diluted to 100 c.c. and preserved by a few drops of toluene or xylene, 5 c.c. diluted to 500 c.c., gives a solution containing 1 mg. of dextrose in 10 c.c. Use 2 c.c. for each determination.
2. An alkaline copper solution made from 40 grams of anhydrous sodium carbonate in about 400 c.c. of water, then pour in a liter flask and add 7.5 grams of tartaric acid and when this has dissolved 4.5 grams of crystallized copper sulphate. Make up the volume to a liter. If a sediment separates use the decanted clear solution.
3. A phosphotungstic-phosphomolybdic acid solution (phenol reagent). In a large flask place 25 grams of molybdenum trioxide or 34 grams of ammonium molybdate, add 140 c.c. of 10 per cent. sodium hydroxide and about 150 c.c. of water. Boil twenty minutes to drive off the ammonia. Then add 100 grams of sodium tungstate, 50 c.c. of 85 per cent. phosphoric acid and 100 c.c. of concentrated hydrochloric acid. Dilute to 700 to 800 c.c., close the mouth of the flask with a funnel and watch glass. Boil gently for not less than four hours adding water to replace that lost. Cool and dilute to a liter. When used for blood sugar determination, dilute 2 volumes with 1 volume of water and 1 volume of concentrated hydrochloric acid.
4. A saturated aqueous sodium carbonate solution.

For the determination of blood sugar heat a beaker of water to vigorous boiling. Transfer 2 c.c. of the protein-free blood filtrate to a test tube (20 mm. x 200 mm.) graduated at 25 c.c. and place 2 c.c. of the standard sugar solution in another similar tube. Add to each tube 2 c.c. of the alkaline copper tartrate solution and heat in the boiling water for six minutes. Remove, and to each tube add while hot 1 c.c. of the acidified and diluted phenol reagent. Mix and cool, then add to each 5 c.c. of the saturated sodium carbonate solution when an intense blue color gradually develops. Dilute to the 25 c.c. mark and after five minutes compare the two solutions in a colorimeter.

Calculate the sugar by multiplying the depth of the standard (in mm.) by 100 and dividing by the reading of the unknown; this gives the mg. of sugar in 100 c.c. of blood.

The Denison Laboratories colorimeter¹ (Fig. 8) can be made at little expense. Select two test tubes of equal diameter holding about 35 c.c. and graduate them from 1 to 30 c.c. in fifths. Remove one end of a microscope slide box and in the other bore two holes three-fourths of an inch apart and the diameter of the tubes. Give a ground surface to a microscope slide by rubbing two together with powdered carborundum or emery in oil between them. Insert this under the tubes. Insert this under the tubes. Have the inside

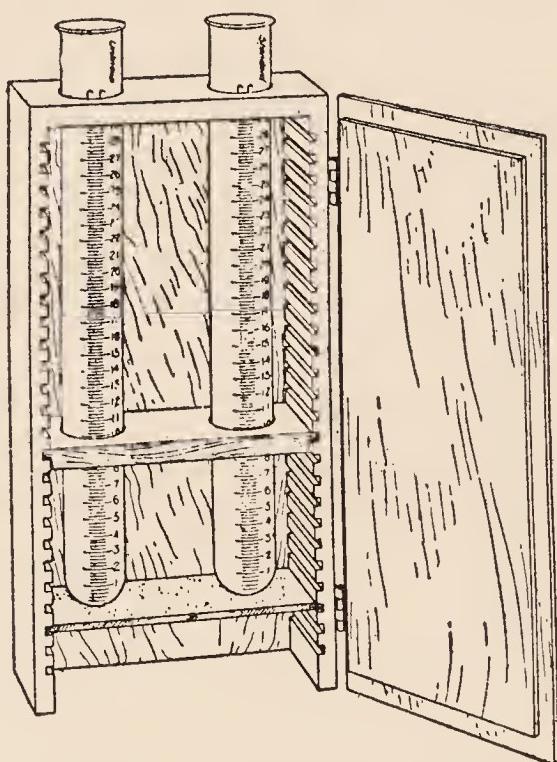


Fig. 8.—Denison Laboratories colorimeter.

of the box painted with a dead black finish and provide as a reflecting surface a mirror, bright tin or white paper.

To use this colorimeter fill one tube with the solution to be tested until the bottom of the meniscus comes to the 10 c.c. mark and in the other put enough of the standard solution to give the same shade when looked at from a distance of about two feet. The solution can be added or removed by a pipette with a rubber bulb or a pen filler. Turning the box around so as to reverse the position of the tubes may increase accuracy, and it is well to take the average of several readings.

¹ Journal of the American Medical Association, 1918, lxx, 679.

Multiplication of the reading on the standard tube by 10 gives the percentage concentration of the unknown as compared with the standard. For example, if the average reading of the standard tube is 7.6 c.c. the concentration of the unknown solution is 76 per cent. of the standard; and if the reading is 11.9 c.c. the unknown is 119 per cent. of the standard.

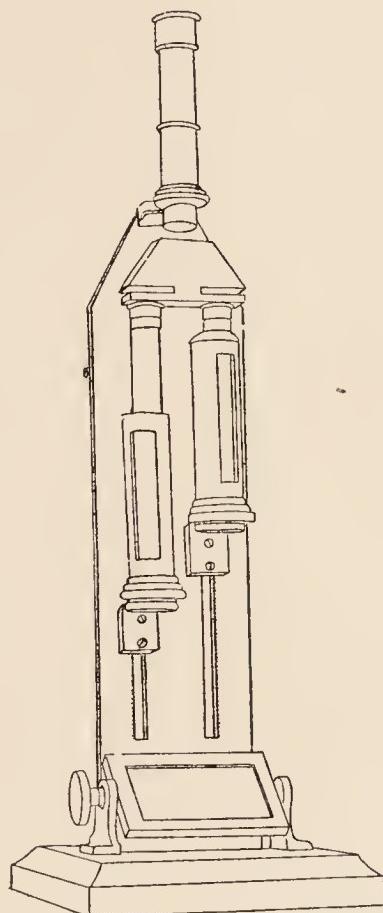


Fig. 9.—Duboseq colorimeter. The depth of liquid in the two cylinders can be changed by raising or lowering them. Light is reflected from the mirror through the liquid, then through glass prisms to the eye-piece which brings the images together.

386. Determination of Non-protein Nitrogen.—This is made by a modification of the micro-Kjeldahl method in which the nitrogen is converted to an ammonium salt by digestion with acid, then the ammonia is Nesslerized.

The acid is a mixture of 300 c.c. of syrupy phosphoric acid (about 85 per cent.) with 100 c.c. of concentrated sulphuric acid. (Calcium sulphate may separate and can be allowed to settle out by standing in a tall, closed cylinder.) To 100 c.c. of this clear

mixture add 10 c.c. of 6 per cent. copper sulphate solution and 100 c.c. of water. The digestion is made in Pyrex ignition glass test tubes, 200 mm. x 25 mm. They should be graduated at 35 c.c. and 50 c.c.

Introduce 5 c.c. of the protein-free blood filtrate into a dry Pyrex test tube, add 1 c.c. of the sulphuric-phosphoric acid mixture and a dry quartz pebble to prevent bumping and boil vigorously over a microburner until the dense acid fumes appear; this usually requires three to seven minutes. Then reduce the flame until the liquid is just visibly boiling and close mouth of the tube with a watch glass; continue the gentle heating for two minutes. If the mixture is not colorless at that time continue heating until it is. Allow the mixture to cool for seventy to ninety seconds, then add 15 to 25 c.c. of ammonia-free water. Cool to room temperature and dilute with water to the 35 c.c. mark. Add 15 c.c. of the Nessler solution, close the tube with a rubber stopper and mix; if the mixture is turbid centrifuge it before comparing with color of the standard.

(The Nessler reagent is an alkaline solution of mercuric potassium iodide. To make it place 150 grams of potassium iodide and 110 grams of iodine in a 500 c.c. Florence flask with 100 c.c. of water and 140 to 150 grams of metallic mercury. Shake the flask vigorously until the dissolved iodine has nearly disappeared. When the hot solution has become a pale red cool it in water and shake until the reddish color has changed to the greenish of the double iodine. Not more than fifteen minutes should be required. Decant the solution from the mercury, washing it off with distilled water. Dilute the clear solution to 2 liters with water. To finish the Nessler reagent use 3500 c.c. of 10 per cent. sodium hydroxide solution with 750 c.c. of the above mercuric potassium iodide solution and 750 c.c. of water.)

The standard ammonia solution can be made by placing in a 100 c.c. measuring flask 3.3 mg. of pure ammonium sulphate (which contains 0.3 mg. of nitrogen) with 50 c.c. of ammonia-free water, and 2 c.c. of the sulphuric-phosphoric acid mixture; fill to the mark and mix. The unknown and the standard should be Nesslerized at about the same time. Compare in a colorimeter. If the standard is set at 20 mm., 20 divided by the read-

ing of the unknown and multiplied by 30 gives the milligrams of non-protein nitrogen in 100 c.c. of blood.

387. Determination of Urea.—Several modifications of the method are possible. A convenient one is to convert the nitrogen of the urea to ammonia by means of urease, distil the ammonia into acid and Nesslerize this.

The Nessler reagent is made as above. The urease solution is made¹ from powdered jack beans freed from ammonia by permutit. In a 200 c.c. flask wash about 3 grams of permutit by decantation, once with 2 per cent. acetic acid and twice with water. Then add 35 c.c. of 95 per cent. alcohol, 70 c.c. of water and 5 grams of jack bean meal; shake for ten minutes and filter. The filtrate contains the urease and will remain good for a week at room temperature, for several weeks on ice. The urease reacts best in presence of a buffer phosphate mixture which regulates the reaction. Such a one can be made from 140 grams of sodium pyrophosphate and 20 grams of glacial acetic acid diluted with water to 1 liter.

Transfer 5 c.c. of the protein-free tungstic acid blood filtrate to a clean and dry Pyrex ignition-glass test tube, capacity about 75 c.c. (If the tubes have been used for Nesslerizing they must be cleaned with nitric acid because traces of mercury destroy urease.) Add to the blood filtrate 2 drops of the buffer phosphate solution described above, then add 0.5 to 1.0 c.c. of the urease solution described. Let the fermentation go on at room temperature for fifteen minutes or more, or let the tube stand for five minutes in a beaker of warm water, not above 55°.

The ammonia formed can be distilled into 2 c.c. of 0.05 normal hydrochloric acid contained in a second test tube of about 50 c.c. graduated at 25 c.c. Close the first tube with a rubber stopper provided with a glass tube about 8 inches long bent downward at an angle of about 45°, the lower end of the tube dipping into the hydrochloric acid in the second tube. The receiving test tube is kept in place by a rubber stopper which slides quite loosely over the bent glass delivery tube; this stopper has a notch cut in the side to allow of escape of steam.

Add to the fermented blood filtrate a dry quartz pebble, 2 c.c. of saturated borax solution and a drop or two of paraffin oil; insert firmly the rubber stoppers and boil moderately fast over a

¹ Or it can be purchased in powdered form.

microburner for four minutes, heating so that the steam does not escape in less than three minutes. At the end of three minutes slide down the receiving test tube so that the bent delivery tube comes but slightly below its mouth and continue the distillation one minute more. Then rinse off the outside of the delivery tube into the receiving test tube with a little water.

Cool the distillate with running water, dilute to about 20 c.c. and add 2.5 c.c. of Nessler's reagent described above. Fill to the 25 c.c. mark and compare in a colorimeter with a standard containing 0.3 mg. of nitrogen (as in the determination of non-protein nitrogen). If the standard is set at 20 mm. multiply 20 by 15 and divide by the height of the colorimetric reading to get the urea nitrogen in 100 c.c. of blood.

387a. Determination of Creatinine.—A standard solution of creatinine is prepared by placing in a liter flask 6 mg. of creatinine (which may be from the standard used in urine analysis) adding 10 c.c. of normal hydrochloric acid, diluting to the mark and mixing. It can be preserved by a few drops of toluene or xylene. Five c.c. plus 15 c.c. of water contains 0.03 mg. of creatinine and is the standard needed for most human bloods.

Transfer 25 c.c. (or 50 c.c.) of a purified saturated picric acid solution to a small clean flask, add 5 c.c. (or 10 c.c.) of 10 per cent. sodium hydroxide and mix; this alkaline picrate solution gives a reddish color with creatinine from the intensity of which the amount can be estimated.

In a small flask place 10 c.c. of the protein-free blood filtrate and in another flask 5 c.c. of the standard creatinine, diluting the latter to 20 c.c. Then add 5 c.c. of the freshly prepared alkaline picrate solution to the blood filtrate and 10 c.c. to the diluted creatinine solution. Test the colorimeter to make certain that both fields are equal when both cups contain the standard solution. After eight to ten minutes make color comparison of the standard and blood solutions, usually setting the standard at 20. This should be completed within fifteen minutes from the time of adding the picrate.

Calculate the amount of creatinine by multiplying the reading of the standard (usually 20) by the volume of the standard taken (here 5 c.c.) and dividing by the reading of the unknown; the result is the mg. of creatinine in 100 c.c. of blood.

387b. Determination of Creatine Plus Creatinine.—Transfer 5 c.c. of the protein-free blood filtrate to a test tube graduated to 25 c.c. such as is used for urea and sugar determinations. Add 1 c.c. of normal hydrochloric acid, cover the mouth of the test tube with tin-foil and heat in the autoclave to 130° for twenty minutes or to 155° for ten minutes. This converts the creatine into creatinine.

Add 5 c.c. of the alkaline picrate solution and at the same time add to 20 c.c. of the standard creatinine solution in a 50 c.c. flask 2 c.c. of normal hydrochloric acid and 10 c.c. of the alkaline picrate solution. After standing eight to ten minutes dilute the blood solution to 25 c.c. and the standard solution to 50 c.c. Set the standard in the colorimeter at 20 and compare with the other solution.

The height of the standard (usually 20 c.c.) divided by the reading of the unknown and multiplied by 6 gives the "total creatinine" (creatinine plus creatine) in mg. per 100 c.c. of blood.

387c. Determination of Uric Acid.—The uric acid is precipitated from the protein-free blood filtrate as silver urate. This is dissolved and treated with phosphotungstic acid which is reduced, yielding a blue color which is compared with that from a standard solution of uric acid.

The solutions needed are:—

1. A standard solution of uric acid is made by dissolving 1 gram of uric acid in 125 to 150 c.c. of 0.4 per cent. lithium carbonate, then diluting to 500 c.c. Transfer 50 c.c. to a liter volumetric flask; add 200 to 300 c.c. of water, then 500 c.c. of 20 per cent. sodium sulphite solution which has been filtered after standing over night. Dilute to the mark and mix; it will keep indefinitely. For daily use portions can be kept in 200 c.c. rubber stoppered bottles. Use 3 c.c. for each determination.

2. A 10 per cent filtered sodium sulphite solution.

3. A 5 per cent. sodium cyanide solution, to be added from a burette (2.5 to 5 c.c. for each determination).

4. A 10 per cent. solution of sodium chloride in 0.1 normal hydrochloric acid.

5. The uric acid reagent is prepared by heating 100 grams of sodium tungstate and 80 c.c. of 85 per cent. phosphoric acid in

700 c.c. of water with a reflux condenser for two hours. Cool and dilute to a liter.

6. A 5 per cent. solution of silver lactate in 5 per cent. lactic acid (4 to 5 c.c. for each determination).

To 10 c.c. of the protein-free blood filtrate in each of two 15 c.c. centrifuge tubes add 2 c.c. of the silver lactate solution and stir with a fine glass rod. Centrifuge and add a drop of silver lactate to the supernatant liquid which should remain almost perfectly clear. Then decant the supernatant liquid as completely as possible.

Add to each tube 1 c.c. of the 10 per cent. sodium chloride in hydrochloric acid and stir thoroughly; then add 5 to 6 c.c. of water, stir and centrifuge. This sets free the uric acid. Decant the two supernatant liquids into a 25 c.c. volumetric flask, add 1 c.c. of 10 per cent. sodium sulphite, 0.5 c.c. of 5 per cent. sodium cyanide and 3 c.c. of 20 per cent. sodium carbonate.

Prepare simultaneously two standard solutions of uric acid as follows: transfer to one 50 c.c. volumetric flask 1 c.c., and to another 50 c.c. flask 2 c.c. of the standard uric acid sulphite solution. To the first flask add 1 c.c. of 10 per cent. sodium sulphite; then to each flask add 4 c.c. of the acidified sodium chloride solution, 1 c.c. of sodium cyanide, and 6 c.c. of the 20 per cent. sodium carbonate solution. Dilute with water to about 45 c.c.

At this stage add to the two standards and the unknown the phosphotungstic acid solution,—0.5 c.c. to the unknown and 1 c.c. to each of the standards and mix. After ten minutes fill to the mark with water, mix and compare in the colorimeter, setting the standard at 20.

When the weaker standard is used multiplication of the reading of the standard (here 20) by 2.5 and dividing by the reading of the unknown gives the milligrams of uric acid in 100 c.c. of blood.

THE BILE.

The bile is normally a brown to greenish, viscid fluid with a bitter taste and a neutral or slightly alkaline reaction. It is a mixture of the secretions of the liver cells

with that from the mucous membrane of the passages, which latter contains a viscous substance similar to the nucleoproteins. This is usually called biliary mucin, although it differs in some respects from true mucin.

The compounds which make up the larger part of the solid matters of the bile are the sodium salts of glycocholic and taurocholic acids. Beside these and the biliary mucin, there are present fats, soaps, lecithin, and cholesterol, also a number of inorganic salts of the alkalies, alkaline earths, and iron. The color of the bile is due to the biliary pigments, bilirubin, biliverdin, etc.

The salts of the biliary acids in the bile of different animals vary in their proportions. In the case of carnivorous animals chiefly the taurocholic acid is found; in the human bile, as well as that of most cattle, both are present. The biliary acids are both compounds of cholic acid, $C_{24}H_{40}O_5$. Glycocholic acid, $C_{26}H_{43}NO_6$, is composed of cholic acid united with glycocoll, $CH_2-NH_2CO_2H$; taurocholic acid, $C_{26}H_{45}NSO_7$, of cholic acid and taurin, $C_2H_4NH_2SO_3H$. They can be decomposed into their constituents by the caustic alkalies. With cane sugar and sulphuric acid the biliary acids give a purple color, and this can be used as a test of their presence.

This test is an extremely delicate one, and its failure indicates that biliary acids are absent. There are, however, other substances—like albumin, morphine, and amyl alcohol—which give a similar color. In these cases the spectroscopic test should not be neglected. The biliary acids can be obtained pure by evaporating the solution to dryness, extracting with absolute alcohol, precipitating this solution with ether, and applying the test to the precipitate. The purple solution, when sufficiently diluted with alcohol and examined spectroscopically, gives a dark band between *D* and *E*, near to *E*, and another before *F*. These are not seen with albumin, etc.

In concentrated sulphuric acid they give a green color, showing a strong fluorescence. The sodium salts are obtainable from the bile by evaporating to dryness and, after dissolving in alcohol, precipitating with ether.

Cholesterol, $C_{26}H_{43}OH$, occurs in most of the fluids of the body, as well as in the bile, and the calculi or concretions of the gall bladder, of which it forms the principal part. It is not common in the urine, but is a constant ingredient of the feces. It is insoluble in water, but soluble in ether, chloroform, or hot alcohol. It crystallizes from ether in fine, silky needles; from alcohol in large plates containing a molecule of water of crystallization. (Plate I, 4.) In large quantities it has the appearance of a mass of white plates with a pearly luster and a greasy feeling. It is distinguished from the fats by its insolubility in the caustic alkalies, even when boiling. It forms compounds with the fatty acids similar to the fats, the cholesterol taking the place of the glycerol. Lanolin, which is found in wool fat, is an example. These are not easily decomposed by bacteria, hence can be advantageously substituted for the animal fats where decomposition is objectionable. The cholesterol as found in the animal body seems rather to be an excrementitious material than to have any function of its own.

The bile contains a number of well-characterized pigments or coloring matters: bilirubin, $C_{32}H_{36}N_4O_6$; and biliverdin, $C_{32}H_{36}N_4O_8$ are the most abundant and best known. The different colors of bile from a brown to a green are due to a preponderance of one or other of these. They are formed from the blood coloring matters, being found in old blood extravasations, and being increased in amount in the bile when the blood corpus-

cles are destroyed, so that the coloring matters are set free in the plasma.

Bilirubin occurs in many biliary calculi, particularly in and around the nucleus. This is the best source of the pure substance. It is commonly an amorphous powder, orange red in color. It is insoluble in water, but can be dissolved in chloroform, and crystallizes from the latter in plates and prisms. It unites with strong bases and in calculi occurs in union with calcium. By reduction hydrobilirubin, $C_{32}H_{40}N_4O_7$, is formed. This change takes place in the large intestine as a result of putrefactive action, and the color of the feces is due principally to the hydrobilirubin. Bilirubin is acted upon by oxidizing agents, with the formation of biliverdin. This change takes place when an alkaline solution is left exposed to the air.

Biliverdin is an amorphous, green powder. It differs from bilirubin in being insoluble in chloroform, and the two can consequently be separated by this reagent.

Both of these biliary pigments when acted upon by yellow nitric acid, such as is formed by allowing the strong acid to stand in a bright light, undergo a change of color through green, blue, violet, and red to yellow.

Beside these two coloring matters a number of others have been described by different authors. Of them comparatively little is known. They appear to be derived from biliverdin and bilirubin, and it is to their formation that the play of colors is due when bile is acted upon by oxidizing agents. Some of these are:—

Biliprasin, greenish black.

Bilifuscin, brown.

Bilicyanin, blue.

Choletelin, yellow to brown.

Not infrequently there are found in the gall-bladder concretions, commonly known as gall-stones. They are sometimes nearly as large as a hen's egg, and may fill the bladder almost completely. They are soft and often worn away from rubbing against one another. If they are cut through, the nucleus is generally found to be dark colored and composed of bilirubin-calcium. Around this are concentric layers, usually of cholesterol, but sometimes of the bilirubin-calcium. Calcium carbonate is also found in the concretions, as well as others of the biliary pigments in smaller amounts.

388. Add a few drops of acetic acid to 2 to 3 c.c. of bile. The biliary mucin is precipitated. Acidify more strongly and the bile acids begin to be thrown down.

389. **Separation of the Salts of the Biliary Acids.**—About 25 c.c. of ox bile should be mixed with washed sand and evaporated to dryness on the water bath. Then pulverize the mass in a mortar, which operation is facilitated by the sand. Dissolve the biliary salts by about 50 c.c. of strong alcohol, and filter. Cool, pour into a flask, and add ether until a precipitate begins to form, shaking to mix thoroughly. After standing a few hours the precipitated mass is converted into clusters of silky crystals (sometimes called crystallized bile). These are a mixture of the sodium salts of the taurocholic and glycocholic acids. They can be purified by filtering, washing with water, dissolving in the smallest possible quantity of water, stirring in a pinch of bone-black, filtering, and precipitating again with ether. They crystallize then in long, thin, colorless crystals with a silky luster.

390. **Pettenkofer's Test for the Biliary Acids.**—Use the salts or the ox-bile. Mix in a porcelain dish or test tube with a small amount of concentrated sulphuric acid,

being careful not to let the temperature rise above 70° C. It must, however, be above 50°. Then add a few drops of a solution of cane sugar, stirring with a glass rod. A red color appears. If too much sugar is added or the temperature is too high the sugar is decomposed by the acid, giving dark brown products, which conceal the red color.

391. The same result may be obtained by adding the sugar to the liquid to be tested, acidifying with dilute sulphuric acid, and dipping into it a piece of filter paper. Allow the paper to dry, or dry it at a moderate heat, to avoid charring. When it is completely dry, the red color appears on the paper. If heated too highly it will be turned black by the acid.

392. Slightly acidify diluted bile in a test tube with acetic acid, then add about its own volume of a 1 per cent. solution of Witte's peptone. A compound of the peptone and bile acids precipitates, insoluble in acetic acid.

393. Examine the spectrum of the colored liquid obtained in 390. There are two absorption-bands: one at *F*, the other between *D* and *E*, near *E*. Sketch these.

394. **Preparation of the Free Biliary Acids.**—Dissolve in as little water as possible, some of the sodium salts obtained in 389. Add dilute sulphuric acid slowly, until a precipitate commences to form, then add a little ether. After standing in the cold until the acid has separated, filter, wash, and recrystallize from a small amount of hot water. This gives the glycocholic acid. Examine with the microscope and sketch.

395. **Preparation of Cholic Acid.**—Add to ox bile one-fifth of its weight of 30 per cent. sodium hydroxide and boil for twenty-four hours, adding more water to replace that which has evaporated. Then saturate the liquid with carbon dioxide,

evaporate to dryness, and extract the mass with strong alcohol. The sodium salt of cholic acid dissolves, as well as some other sodium compounds. Dilute with water until the solution contains no more than 20 per cent. of alcohol, then precipitate with dilute barium chloride until the reaction is complete. Filter and test the filtrate with barium chloride, which must give no precipitate. Then precipitate the cholic acid from this filtrate by decomposing its sodium salt by means of hydrochloric acid. Let it stand several hours until it has become crystalline, then recrystallize from alcohol. Make sketches of the crystals.

396. Test the cholic acid thus obtained with concentrated sulphuric acid, and notice that it gives a green fluorescence. Add a few drops of a cane sugar solution and see that a red color appears, as with the undecomposed biliary acids.

397. **Preparation of Taurocholic Acid.**—The bile of dogs is preferable to ox bile for this purpose. It contains the sodium salt of the acid. In the following operations use the minimum quantities of solvents. Evaporate the bile to dryness on a water bath, dissolve in alcohol and precipitate with ether as in 389. Dissolve the precipitate in water and precipitate the glycocholic acid by the repeated additions of small amounts of ferric chloride, each time nearly neutralizing the acid reaction with sodium hydroxide. Filter and precipitate the iron from the filtrate by an excess of sodium carbonate. Nearly neutralize the filtrate and evaporate to dryness. Dissolve in absolute alcohol, evaporate to dryness and dissolve in water. From this precipitate the sodium taurocholate by saturating the liquid with sodium chloride. Filter and add hydrochloric acid until the solution contains 2 per cent. If a precipitate of salts appears remove it by filtration and from the filtrate precipitate the taurocholic acid with ether. It should be filtered immediately and can be recrystallized in the same manner, being then obtained in needles or prisms. Its preparation is more difficult than that of the glycocholic acid.

398. Add the solution of the taurocholic acid acidified with sulphuric acid to solutions of albumin or peptones and observe that they form insoluble compounds.

399. Preparation of Taurin.—Mix dog bile with an excess of concentrated hydrochloric acid, and evaporate the liquid to a small volume by boiling. Pour off the solution from the resinous mass of acids which have separated, and evaporate this liquid until the sodium chloride has, for the most part, crystallized out. Filter, and evaporate the filtrate to dryness. From the residue dissolve the glycocoll with alcohol, then the insoluble taurin in the smallest possible quantity of hot water. On cooling the taurin crystallizes out in four-sided prisms.

400. Preparation of Cholesterol.—Biliary calculi, or gall-stones, are the best source of cholesterol. Powder the calculus, remove the bile by boiling water, then dissolve the cholesterol in boiling alcohol, and filter while hot. It separates from the filtrate on cooling. The insoluble residue, which consists largely of compounds of the biliary coloring matters, can be used for the preparation of these (406). The cholesterol may be further purified by dissolving it in an alcoholic solution of sodium hydroxide with the aid of heat. After it separates on cooling, wash well with water on the filter, then recrystallize from a mixture of alcohol and ether.

401. Examine the crystals under the microscope. They are in the form of large rhombic tables or plates. Sketch them.

402. To a crystal of cholesterol in a test tube or under the microscope add a drop of concentrated sulphuric acid, then a drop of iodine solution. The crystal becomes first violet, then blue, green, and red.

403. Dissolve a crystal of cholesterol in a few drops of chloroform in a test tube, then add an equal volume of concentrated sulphuric acid. The chloroform solution becomes red, then cherry-red, and purple. On pouring it into a dish it becomes blue, green, and finally yellow.

404. Evaporate on a piece of porcelain a small crystal of cholesterol with a drop of concentrated nitric acid. A yellow stain remains which, if treated while warm with ammonia, gives a red color. Too high heating prevents the reaction.

All these reactions can be employed for the identification of cholesterol.

405. **Preparation of the Biliary Pigments.**—If biliary calculi are not available, bile may be used for obtaining bilirubin, employing the yellow or brown in preference to the green. Dilute it with a little water and add a small amount of lime water, avoiding an excess. Mix by shaking. Pass through the liquid a stream of carbon dioxide to convert any excess into calcium carbonate. Filter out the bilirubin, which has been precipitated as the calcium compound, and wash it with water. Suspend the precipitate in water, decompose it with a slight excess of hydrochloric acid, and shake it immediately with a small amount of chloroform to take up the free bilirubin, otherwise it will oxidize to biliverdin. Separate the chloroform solution from the water and precipitate the bilirubin from it by alcohol.

406. If biliary calculi are at hand they may be used instead of the bile. Pulverize the calculi, then dissolve the cholesterol with a mixture of alcohol and ether. The residue from the alcoholic extraction in the preparation of cholesterol may be used (400). After the cholesterol has been removed decompose with acid and proceed as in the preceding experiment (405).

407. Convert a portion of the bilirubin into biliverdin by dissolving in dilute sodium hydroxide and letting the

solution stand in an evaporating dish. When it has turned green, precipitate with an excess of hydrochloric acid, filter and wash.

408. The biliverdin in an impure state can be obtained from ox bile by precipitating the mucin with several times its volume of alcohol, then precipitating the biliverdin by barium chloride. Filter, wash with water, and alcohol, then decompose with hydrochloric acid. The biliverdin is insoluble in the acid. To remove the fat it must be extracted with ether, then the biliverdin can be dissolved in alcohol, which, after filtering, is left to evaporate.

409. **The Nitric Acid Test** (Gmelin's test).—To a solution of bilirubin in dilute alkali add slightly yellow, concentrated nitric acid, holding the tube in a slanting position and pouring slowly so that the acid flows down under the bilirubin solution. Notice the colored rings: green nearest the top, then blue, violet, red, and yellow next to the acid. The acid must not be too yellow or the pigments quickly oxidize and nothing is seen but a yellow color.

410. In a porcelain dish place about 5 c.c. of Hammarsten's reagent (1 volume of nitric acid and 19 volumes of hydrochloric acid, each of 25 per cent. When it has become yellow by standing dilute with 4 volumes of alcohol). Add a few drops of diluted bile: a green color appears, with colors as in Gmelin's test if more bile is added.

411. **The Iodine Test** (Huppert's test).—To an alkaline solution of bilirubin add lime water to precipitate the bilirubin. Filter, wash with water, place in a test tube half full of alcohol slightly acidified with sulphuric acid, and boil for some time. The bilirubin is oxidized to biliverdin and the alcohol becomes colored green or bluish green.

412. To a little of the bilirubin solution in a test tube add a very dilute tincture of iodine so that it floats on top. An emerald green ring is seen between the liquids. Explain the action of the iodine.

CONNECTIVE TISSUES.

Beside the areolar tissue, which may be the white, fibrous, or the yellow, elastic, there are cartilaginous and osseous tissues.

WHITE FIBROUS TISSUE.

Nearly pure white fibrous tissue is found in the tendons, which are made up of fibers, matrix, and cells. The fibers consist of collagen; the matrix contains mucoid; the cells contain proteins and salts.

413. Chop a tendon finely (the tendon of Achilles can be used) and remove the soluble constituents by washing with cold water. Extract the mucoid with lime water and filter. Preserve the insoluble residue and use the solution for the mucoid tests.

414. Show that mucoid gives the biuret and Millon's reactions.

415. It cannot be coagulated by heating nor does it dissolve in dilute acids (distinction from most other proteins).

416. It does not reduce Fehling's solution unless it has been boiled for an hour or two with an acid like hydrochloric. A reducing compound is thus produced. When the acid solution is neutralized a precipitate of acid metaprotein falls.

What has been proved as to composition of mucoid?

COLLAGEN.

417. For its preparation use the tendon from which the mucin was removed. Heat the tendon with a little water for several hours. On cooling it gelatinizes and gives the gelatin reactions (page 63).

CARTILAGE.

418. This contains collagen mixed with chondrogen, chondroitin sulphuric acid, and an albuminoid. It can be obtained from pigs' tracheas. Separate the surrounding tissues, grind the remainder and boil several hours with a little water. The filtered solution yields a jelly when it cools (gelatin from the hydration of the collagen).

419. The solution gives no reaction for sulphuric acid with barium chloride, or at most but a slight one, but does so after boiling for some time with hydrochloric acid, showing that it contains an organic sulphate, chondroitin sulphuric acid.

420. The solution reduces Trommer's or Fehling's reagent after boiling some time with hydrochloric acid, then neutralizing, but not before. It gives the protein reactions.

What would these three experiments show about the constituents of cartilage?

BONE.

Bone contains an organic compound, collagen, or ossein, and a number of inorganic or mineral substances. These latter are the phosphates of calcium and magnesium, mainly the former; also calcium carbonate and small amounts of calcium chloride and fluoride. The

inorganic substances can be removed by acids, leaving the bone flexible. If a bone is heated the collagen is decomposed, with an evolution of ammonia, showing that the collagen is a nitrogenous compound. Then inflammable gases are set free. If the ignition is performed where free access of air is prevented, there remains a black mass known as bone black, or animal charcoal. The black color is due to carbon, which can be removed by burning in the air, leaving the mineral or inorganic constituents only. The bone black is an extremely porous substance and has the power of absorbing from their solutions many of the vegetable coloring matters and also the alkaloids. On this account it is used for decolorizing liquids as well as for an antidote in cases of poisoning by strychnine and some other alkaloids.

421. Fill a dry test tube one-third full of fragments of dry bone, fasten it horizontally by the upper end in a clamp, and heat, at first gently then to as high a temperature as possible without softening the glass, moving the burner so as not to heat it in one spot. The organic matter is decomposed. First water is given off, then ammonia. Test this with a piece of red litmus paper. An oily or tarry mixture distils off with inflammable gases. When the gas has been expelled the mineral matters of the bone remain mixed with carbon.

422. Take a piece of this and heat it in the air, holding it with the forceps or a piece of wire. The carbon burns away, leaving only the mineral matters as a brittle mass.

423. Dissolve the bone ash in dilute nitric acid. Notice that carbonates are present, as shown by the effervescence of carbon dioxide gas.

424. Test a portion of the solution for the PO_4 radical by making it strongly acid with nitric acid, then adding ammonium molybdate and warming gently. A yellow precipitate shows that the phosphate radical is present.

425. Test another small portion of the solution for chlorides with silver nitrate. They give a milkiness or a white precipitate, but are present in very small quantities.

426. Test the remainder of the nitric acid solution for calcium and magnesium after removing the phosphoric acid in the following manner: Add a few drops of ferric chloride to the solution in a beaker. The iron unites with the combined phosphoric acid and forms ferric phosphate. Pour a few drops from the beaker into a test tube and test by adding ammonia to see if all the phosphoric acid has united with the iron. If this is the case, the ammonia gives a yellowish precipitate. If not enough ferric chloride was added the precipitate is white, and more of the iron solution must be added and the test repeated until all the phosphoric acid has been taken by the iron. Then to the solution add a solution of sodium carbonate until it is nearly neutral; that is, until the precipitate—which forms as the sodium carbonate strikes the liquid—dissolves slowly on stirring. Then add 1 or 2 grams of barium carbonate to precipitate the ferric phosphate. Filter after warming. Precipitate the barium from the hot filtrate with dilute sulphuric acid and filter. Having thus removed the phosphoric acid, test the filtrate for calcium by making it alkaline with ammonia and adding ammonium oxalate as long as a precipitate is formed. The calcium is thrown down as white calcium oxalate. Filter and to the filtrate add

sodium phosphate. A white crystalline precipitate shows magnesium.

427. Test the absorptive power of bone black after it has been pulverized by adding it to a light blue solution of indigo and warming, then filtering. The coloring matter will have almost or entirely disappeared from the filtrate.

Directions have been given before for the separation of the collagen from the mineral constituents of the bone.

427a. What substances have been shown to be contained in bone?

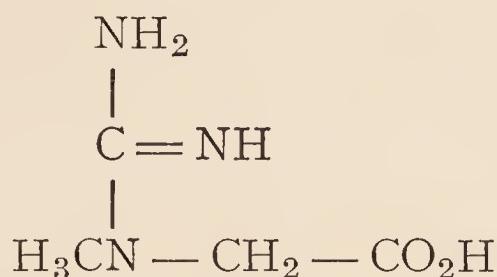
MUSCULAR TISSUE.

A muscle is made up of fibers or cells, consisting of a sheath (the sarcolemma), composed of a substance similar to elastin, and its contents. The latter are mostly albuminous matters, alkaline and liquid during life, but becoming acid and more solid after death. This albuminous liquid, which in many respects corresponds to the plasma of the blood, is called muscle-plasma. It coagulates quickly after death at the ordinary temperatures, and thus gives rise to the *rigor mortis* of the muscles. The coagulated mass is myosin, a globulin.

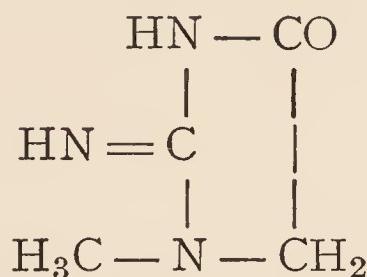
If fresh muscular tissue is treated with boiling water, most of the albuminous substances are coagulated, and upon filtering remain with the fats in the insoluble residue. The solution contains, beside inorganic matters, a class of organic compounds, sometimes called, from the method of obtaining them, the "extractives." They may be divided into two groups: those which contain no nitrogen and those of which nitrogen is a constituent. Among the non-nitrogenous are lactic acid and its compounds, also glycogen and its derivatives: dextrin, maltose, and glucose. The principal ones of the nitrogenous extract-

ives are creatine and creatinine, small quantities of urea and uric acid, and the nuclein bases, such as guanine, xanthine, and hypoxanthine—formerly called sarcine. Carnine, which is similar in properties and composition to the nuclein bases, is also found in the watery extract of muscle.

Creatine,

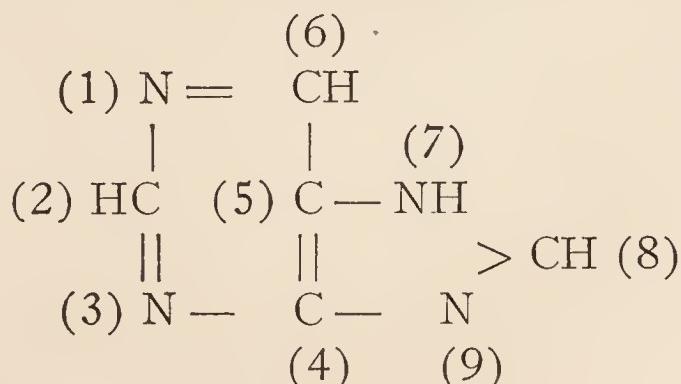


and creatinine,



are closely related, the latter being derived from the former by taking away one molecule of water, and can be changed back into creatine by adding the water again.

The nuclein bases (also called purine bases, xanthine bases, and alloxuric bases), guanine, $\text{C}_5\text{H}_5\text{N}_5\text{O}$; hypoxanthine, $\text{C}_5\text{H}_4\text{N}_4\text{O}$; and xanthine, $\text{C}_5\text{H}_4\text{N}_4\text{O}_2$, are similar in their properties and related to uric acid, $\text{C}_5\text{H}_4\text{N}_4\text{O}_3$. They are derivatives of the hypothetical nucleus, purin:



They are formed by the addition to, or substitution in this of oxygen or organic radicals, the position being indicated by the figures. Thus guanine is 2-amino-6-oxypurin. Hypoxanthine is 6-oxypurin; xanthine, 2,6-dioxypurin and uric acid, 2,6,8-trioxypurin. They occur partly free in the muscular tissue and partly united with phosphoric acid and albuminous substances in the form of nucleins. All these soluble compounds are found, naturally, in the various meat extracts which are used in foods. They are probably formed in the body by the decomposition of the nuclein compounds. When taken as a food, their value is rather in the stimulation of digestion through their agreeable taste than in their absolute nutritive worth. This may be merely because of their increasing the secretion of the digestive fluids.

428. Pith a frog (430): cut out the gastrocnemius muscles. Let one remain in water at a temperature of 50° until *rigor caloris* has appeared. Put the other into boiling water. Test the reaction of both with litmus paper. The former is acid: the latter is alkaline.

429. Kill a rabbit or a frog; at once lay bare the muscle and test it with red and blue litmus paper and with lacmoid paper. To the latter it is neutral; the red litmus turns blue and the blue litmus, red—that is, the reaction toward litmus is amphoteric. Both free acids and acid salts turn litmus red; lacmoid becomes red from the action of free acids, not from that of the acid salts. Let the muscle stand and test it as before. It remains neutral to lacmoid, but is acid to litmus. Lactic acid has been produced, but instead of remaining in the free state it forms lactates. The bases are thus removed in part from other salts, leaving them as acid salts (for

example, acid phosphates), which latter produce the acid reaction.

430. Inject a solution of acid fuchsin into the subcutaneous lymph-space of a frog. After it has been absorbed pith the animal; that is, destroy the brain by pushing forward a stout wire inserted through the occipital-atlantoid membrane. This lies in the middle of a line drawn across the back of the head through the posterior margin of the tympanic membranes. Strip the skin from both hind legs and separate the muscles of one thigh until the sciatic nerve is exposed. Hang the frog by a hook through the jaws and repeatedly stimulate the sciatic nerve by electrodes passing under it. The stimulated leg contracts vigorously while the other remains passive. In the working muscle lactic acid is formed and this decomposes the colorless, alkaline salt of fuchsin, producing a pink or red color. In the resting leg there is no such acid formation and the color remains pale.

431. Chop finely 20 to 25 grams of lean meat and extract it for an hour with three times as much cold water, stirring frequently. Filter through muslin and test the filtrate for myogen. It is coagulable and gives the general reactions for simple proteins (page 45). It is soluble in distilled water and does not precipitate from this solution on dialysis.

432. Soak the residue from the last experiment in 50 to 75 c.c. of 10 per cent. ammonium chloride solution, filter through muslin and test the filtrate for a globulin, myosin, sometimes called paramyosinogen or muscularin. It responds to the globulin tests (page 58).

433. Mince finely 10 to 15 grams of fresh muscular tissue and extract it by stirring with water for a few minutes. Warm the filtered solution in a double beaker (155). When coagulation occurs filter and keep the temperature constant until no further change is observed, then increase it. Note the temperatures at which the different proteins coagulate and report them. This is the method of separation by fractional coagulation.

434. Preparation of Muscle-plasma.—Kill a frog and immediately wash the blood from the body by passing through a cannula inserted in the aorta a cold 0.5 per cent. sodium chloride solution. The necessary force can be gained by placing the solution in a doubly tubulated bottle, which can be raised and lowered, and connecting the lower tubulure with the cannula by a small rubber tube. Cut the muscle up as quickly as possible with a cold knife or pair of scissors and freeze it by stirring in a beaker, previously surrounded by a freezing mixture of ice (3 parts) and salt (1 part). It freezes at about -7° C. Then rub it to as fine a powder as possible in a mortar, which, as well as the pestle, has been cooled below this temperature by standing in a freezing mixture. Subject the mass to a strong pressure, which gives a yellowish liquid. Filter this through muslin at a temperature below freezing. The filtrate is the muscle-plasma. Through the whole process care must be taken to preserve a low temperature to prevent coagulation.

435. Pour a few drops of the plasma into a dish of the ordinary temperature. It coagulates immediately.

436. Test the reaction of the plasma to litmus paper. It is alkaline.

437. Allow the temperature of the rest of the plasma to rise slowly, and notice that it coagulates at a little above freezing. On standing, a yellowish liquid is pressed out of the clot, as in the case of the coagulum of blood-plasma. This is muscle-serum.

438. Try the reaction of the muscle-serum to litmus paper. It is alkaline.

439. Prove that the coagulated mass is a globulin (163, 164, 166).

440. Take about 500 grams of lean beef, and, after removing, as completely as possible, the fat and connective tissue, chop it finely. Add an equal weight of water and heat half an hour on a water bath to 55° or 60° . Filter through muslin, pressing out the water with the hands. Repeat the extraction with half as much water. Unite the filtrates, and boil to precipitate the coagulable proteins. (Instead of the meat a jar of beef-extract can

be used after dissolving in water.) Filter and add lead acetate as long as a precipitate forms to precipitate inorganic salts, avoiding a great excess. Filter and remove the lead by passing hydrogen sulphide gas into the solution. Filter out the lead sulphide and evaporate the filtrate on the water bath to 5 or 10 c.c. Allow the yellowish syrupy liquid to stand two or three days in a cool place, when the creatine crystals will separate. Filter, and wash with 88 per cent. alcohol. Unite the filtrate and the washings and remove the alcohol by evaporation on a water bath. After cooling make alkaline with ammonia and add an ammoniacal solution of silver nitrate. Filter. The precipitate contains the silver compounds of *hypoxanthine*, *xanthine*, and *guanine*. (The filtrate contains lactic acid. Preserve for testing.) Wash with ammonia and dissolve in boiling HNO_3 , sp. gr., 1.1, to which a little pure urea has been added to prevent the decomposition of the bases. While hot, filter from a small amount of AgCl , which may remain, then allow to stand twelve hours. *Hypoxanthine-silver nitrate* separates in small needle shaped crystals. Filter and wash with water. From the filtrate, by the addition of an excess of ammonia, is obtained a slight precipitate of *xanthine-silver oxide*. The free xanthine and hypoxanthine may be obtained by suspending their silver compounds in water and, after heating and making slightly alkaline with ammonia, adding ammonium sulphide drop by drop until the silver is precipitated, avoiding an excess. On evaporating the filtrate the xanthine and hypoxanthine will be left as microscopic crystals.

Most of the guanine is left in the precipitate made by the ammonium sulphide. It can be dissolved by boiling with a little very dilute hydrochloric acid. Filter and precipitate it from the filtrate by making it alkaline with ammonia.

To obtain the lactic acid from the filtrate from the precipitated hypoxanthine, etc., first precipitate the silver by H_2S and filter. Concentrate the filtrate on the water bath until most of the ammonia has been expelled. Then cool and acidify strongly with dilute sulphuric acid. The lactic acid is thus set free and can now be separated by shaking gently with about one-fifth of its volume of ether, which dissolves the lactic acid, but not the sulphuric. After shaking in a glass-stoppered funnel, allow it to

stand until the ether has all risen to the top of the liquid. Then draw off the water and the ether into separate flasks. Repeat the operation a few times with fresh portions of ether. Mix the different portions of ether and distill or evaporate it. The residue contains the lactic acid mixed with a little sulphuric. Dilute with water and boil a minute with zinc carbonate until it has lost its acid reaction. Filter, and evaporate the filtrate on the water bath to a small volume. Then let it stand, and the zinc lactate will crystallize in four-sided prisms: $Zn(C_3H_5O_3)_2 + 2H_2O$. Filter these from the remaining liquid, and dry on filter paper.

To obtain the free acid dissolve some of the crystals in water and precipitate the zinc with hydrogen sulphide gas. Filter, and evaporate the filtrate. The acid will be left as a syrupy liquid. Test it for its acid reaction and sour taste. It differs from fermentation lactic acid in that it rotates the plane of polarized light toward the right. Fermentation lactic acid does not do this.

441. Convert a part of the creatine into creatinine by boiling fifteen minutes with very dilute sulphuric acid. Neutralize the acid by adding powdered barium carbonate as long as it effervesces. Evaporate to dryness on a water bath and extract the creatinine from the residue with strong alcohol. Upon evaporating, the creatinine is left in the form of crystals.

442. Dissolve a little of the creatinine in a small amount of water, add a solution of zinc chloride, and allow to stand. Characteristic crystals in clusters or rosettes appear. They are a double salt of creatinine and zinc chloride.

443. To a creatinine solution add a few drops of a freshly-prepared solution of sodium nitroprusside, then, drop by drop, dilute sodium hydroxide. The liquid becomes ruby red, soon changing to straw color. If it is now strongly acidified with acetic acid and boiled, it becomes green, then blue. This is Weyl's test.

444. To a solution of creatinine add a few drops of sodium hydroxide, then of picric acid solution. A red color is obtained. This is Jaffe's test.

445. What would be the effect in roasting beef upon each of the constituents studied above? In what form and where would they be in a stew? In beef extract?

446. With a needle tease out some shreds of muscle from a recently killed frog. Place them on a microscope slide and expose for a few minutes to ammonia gas from a strong solution of the hydroxide. When covered with a cover glass and examined they are seen to contain stellate crystals of ammonium magnesium phosphate, NH_4MgPO_4 . Explain its production. Sketch.

THE BRAIN.

447. Clean the brain (of such an animal as a sheep, dog, pig or calf); pulp it in a mortar and extract it for several days with cold ether. Lecithin and cholesterol are dissolved. Filter and to the filtrate add acetone to precipitate the lecithin. Filter and evaporate the ether-acetone filtrate (away from lights and fires) to obtain cholesterol.

A. Confirm the lecithin by the tests previously given (117, 118).

B. Examine the cholesterol with the microscope for the crystalline form. If it is sufficiently pure try tests (402, 403, 404).

448. Extract the cerebrin from 50 to 100 grams of pulped brain with twice its weight of 95 per cent. alcohol, heating for an hour on a water bath in a flask with a reflux condenser. Filter the solution while hot and let it stand over night. Filter off the cerebrin (which will contain some lecithin and cholesterol).

A. Examine with a microscope. It should be in needle shaped crystals.

B. Try Molisch's reaction with the cerebrin (39).

C. Verify the result by boiling a portion in a flask for an hour with dilute hydrochloric acid, adding water to keep up the volume; then neutralize with sodium hydroxide and try Trommer's reaction. (40).

Galactose is the constituent of the cerebrin which gives these two reactions. To what class of compounds does it belong and why does it react so?

D. Show that cerebrin contains nitrogen by heating, after mixing with powdered soda lime, in a test tube held horizontally with an iron clamp on a stand. Test the escaping gas with red litmus paper. Explain reaction.

E. Show that it does not contain phosphorus, if pure (219).

Summarize information gained experimentally as to the constituents of the brain.

MILK.

The solids of milk are partly dissolved and partly in suspension in the liquid. Of the dissolved constituents the most important are milk sugar, and albumin, a globulin, and some mineral salts. Among the suspended compounds are casein, fat, and calcium phosphate. The average amount of solids in normal cows' or human milk is 12 or 13 per cent. by weight. The reaction of fresh cows, or human milk is nearly neutral, or may be *amphoteric* to litmus; that is, it turns red paper blue and blue paper red.

The specific gravity should be between 1.029 and 1.033 at 15°, and of milk which has been skimmed after standing twenty-four hours it should be between 1.0325 and 1.0365. Thus the removal of fat raises the specific gravity and the addition of water lowers it. The average percentage composition of milk is given by König as follows:—

Cows,—water, 87.17; proteins, 3.55; fats, 3.69; lactose, 4.88; mineral matter, 0.71.

Human,—water, 87.41; proteins, 2.29; fats, 3.78; lactose, 6.21; mineral matter, 0.31.

Casein, which is a phosphoprotein, is not in true solution in milk, since it can be filtered out by unglazed porcelain, though not by filter paper. It is precipitated by

weak acids, as is seen when the milk becomes sour, but is not coagulated by boiling. Rennin breaks up the casein into two compounds: a proteose and an insoluble calcium compound (paracasein calcium, or cheese). Coagulated human casein is not as hard as that of cows. The difference may be partly due to its chemical composition, but largely to the fact that cows' milk contains more casein and calcium and less lactalbumin than human milk. It can be made to form a soft and spongy coagulum similar to the human by dilution or by the removal of the calcium compounds.

The fats of milk are a mixture of stearin, palmitin, and olein, with a small amount of the glycerides of some lower members of the fatty acid series,—butyric, caproic, caprylic, capric, etc. The fat exists as an emulsion, a coating of albumin keeping the globules separate. They may be made to collect by dissolving this coating by a chemical agent, like sulphuric acid. Babcock's method for determining the percentage of fat in milk is based upon this principle. Here the volume of the fat is measured and this gives the relative amount in the milk. If the fat rises for twenty-four hours without such decomposition it should form a layer 10 or 15 per cent. of the depth of the milk, if the latter is normal.

The method of obtaining the milk sugar has been given (63).

449. Test the reaction of fresh milk to red and blue litmus paper and to phenol-phthalein. What is shown?

450. Determine the specific gravity with an accurate urinometer.

451. Remove the fat from milk by a centrifuge, or after standing, and determine the specific gravity again.

Examine again with a microscope. Has all the fat been removed?

452. Try the specific gravity test after adding from 10 to 25 per cent. of water to the milk.

453. How is the specific gravity related to the composition of milk?

454. In a weighed porcelain or platinum crucible evaporate 5 c.c. of milk to dryness on a water bath and weigh quickly to find the amount of the total solid matter. The drying will take place much more rapidly if a weighed quantity (about 20 grams) of dried sand is added, but the residue cannot be used for the next experiment.

455. Heat the dried substance in the crucible, at first gently, then until no black remains. The residue is the mineral matter, or ash. Weigh it. There should not be over 1 per cent. of the weight of the milk.

456. Compare the action of rennin upon cows' and human milk. What is the difference in the nature of the curds? Try the rennin in cows' milk to which 50 per cent. of water and a few drops of ammonium oxalate have been added to remove the calcium salts. How does the absence of calcium salts affect the coagulation?

457. To separate the nitrogenous constituents of milk first precipitate the casein by saturating the milk (skimmed milk can be used) with sodium chloride. Filter, and to filtrate add powdered magnesium sulphate as long as it dissolves, stirring meanwhile. This precipitate is paraglobulin, the same compound that is found in the blood. Filter and apply the globulin tests (163 to 166). Acidify the filtrate with a few drops of dilute acetic acid and boil. The albumin of milk—lactalbumin—is coagulated.

To determine the quantity of protein subtract the sum of fats, sugar, and ash from the total solids.

458. **The Determination of Casein in Milk** (Van Slyke and Bosworth's method).—This is based upon the principle that casein is acid in its nature and forms neutral, salt-like compounds with bases; these can be decomposed by weak acids, yielding a salt of the base and weak acid, together with insoluble casein. An excess of acid is to be used and the precipitated casein is removed by filtration. The excess of acid remaining in the filtrate is determined by titration. The difference between this amount and the quantity added represents that which has united with the base or bases formerly combined with the casein: consequently it corresponds to the amount of casein present.

Measure exactly 17.5 c.c. of milk into a 200 c.c. graduated flask, and add about 80 c.c. of water. Put in a few drops of an alcoholic solution of phenolphthalein for an indicator; then, to neutralize, drop in dilute sodium hydroxide while stirring, until a permanent pink color appears, but avoiding an excess of the hydroxide. Now run in from a burette decinormal acetic acid (6 grams per liter) in portions of 4 to 5 c.c., shaking after each addition and observing whether the curds begin to form upon standing a few seconds. This does not usually occur until after 25 c.c. have been thus added. After 25 c.c. have been used it is better to add but 1 c.c. at a time. When enough acid is present the casein separates in large white flakes, leaving the supernatant liquid clear, not milky. The temperature should be between 18° and 24°. Fill the flask to the mark with water, shake to mix thoroughly, and pour upon a dry filter; the filtrate

should be clear; if it is not the operation should be repeated. Take 100 c.c. of the filtrate and titrate it with decinormal sodium hydroxide until it has been neutralized, as shown by the pink color which the phenol-phthalein present imparts.

Since the concentration of the acid and alkali is equal, the volume of sodium hydroxide used in the last titration represents the excess of acetic acid over what was required to set free the casein from its compound in the milk.

The difference between the volume of decinormal acid used to precipitate the casein and the volume of decinormal alkali required to neutralize the excess corresponds to the amount of casein, and with this volume of milk each c.c. represents 1 per cent. of casein. Before finding this difference the amount of acid used is divided by 2, because but half the acid solution was used for neutralization.

For example, if for 17.5 c.c. of milk were used 30 c.c. of standard acid, and afterwards, to neutralize the excess in 100 c.c. of the filtrate, 11.95 c.c. of the standard alkali, then

$$\frac{30}{2} - 11.95 = 3.05 \text{ per cent. of casein.}$$

459. Examine with the microscope a drop of milk under a cover glass.

460. Destroy the emulsion by mixing 10 c.c. of concentrated sulphuric acid with an equal volume of milk. Let it stand, and the fat rises to the top in large globules. The separation is complete in a few minutes if a centrifuge is used. The volume of the fat can be better seen by using a narrow-necked flask and, after mixing with the acid, nearly filling with warm water.

461. How does the acid act to destroy the emulsion? Is the fat attacked by the acid?

462. Fill a 100 c.c. graduated cylinder to the upper mark with milk and let it stand twenty-four hours. There should be 10 or 15 c.c. of cream.

463. To Determine the Percentage of Lactose in Milk.—Dilute 20 c.c. of milk to 400 c.c. with water. Drop in acetic acid slowly until it coagulates; then pass carbon dioxide gas into the liquid fifteen minutes and let it stand until it settles clear. Filter and wash; coagulate the albumin and globulin in the filtrate by boiling. Filter, wash, and use the filtrate or an aliquot part of it for the sugar determination by Fehling's solution as in the determination of glucose (58). For every 10 c.c. of the solution which is decolorized 0.067 gram of lactose is present or use Folin and McEllroy's method (60).

464. How can it be proved that the sugar of milk is lactose and not dextrose or sucrose?

465. Babcock's Method for the Determination of Fat in Milk or Cream.—When sufficient milk is available, 17.6 c.c. may be taken for each determination. The bottles in which it is treated have a special scale etched upon the rather long neck.

Mix the milk thoroughly by pouring it several times from one vessel to another. With a pipette graduated at 17.6 c.c. measure the milk and pour it into the graduated flask, adding the same volume of 90 per cent. sulphuric acid (sp. gr., 1.82). Mix by gently shaking until the curd has completely dissolved, then revolve in the centrifuge at 600 to 800 revolutions per minute for six or seven minutes. Always make duplicate tests, placing the two bottles opposite each other in the machine. Now carefully fill the bottles about to the highest graduation with hot water, which should have been previously made ready, and whirl again for one or two minutes. Holding the bottle in a perpendicular position, read on the scale the differences between

the upper and lower margins of the fat which gives the percentage present in the milk. If the test is successful the fat layer is clear or but slightly cloudy.

With breast-milk where the available amount is frequently limited smaller flasks and less milk may be employed. Cream must be diluted 5 to 10 times with water.

It is occasionally desired to test milk to learn whether it has been heated. Tests are based upon the fact that unboiled milk contains enzymes (peroxidases) which aid hydrogen peroxide in the oxidation of certain compounds. These enzymes are destroyed if the milk is heated above 80°.

466. Dilute about 1 c.c. of milk with 10 of water, add a few drops of a dilute alcoholic guaiacum solution and let it stand a few minutes. If no blue color appears add a little dilute hydrogen peroxide or old oil of turpentine. Compare with the guaiac test for blood pigments (354).

Compare the results with raw milk and that which has been heated to boiling or has been kept at 80° for 15 minutes.

467. Add to 10 c.c. of the milk in a test tube 1 to 2 c.c. of an alcoholic solution of benzidine, then 2 to 3 drops of acetic acid (or enough to produce coagulation) and lastly let 2 to 3 c.c. of a 2 per cent. solution of hydrogen peroxide run down the side of the tube below the milk mixture. A blue ring appears immediately with the unboiled sample and no blue with the boiled one. Explain.

THE URINE.

The urine is a solution which contains the final products from the chemical changes in progress in the animal body. A part of these are excreted in the expired air and from the skin, and a still smaller part through the mucous membrane of the intestine, but, if we omit the carbon dioxide, eliminated by the lungs, by far the greater proportion of these final products is found in the urine. A study of its composition and variation,

therefore, is often of great value in judging of changes which are going on in the body.

Among the most common inorganic constituents normally found are the chlorides, sulphates, and phosphates of sodium, potassium, calcium, and magnesium. Of the normal organic compounds there are urea, uric acid and its salts, creatinine, etc. The following, when found in more than minute amounts, may be regarded as pathological: Glucose, albuminous substances, blood, bile, pus, fat, mucin, leucin, and tyrosin. Others which are more rare will be spoken of later. All of these either are taken as such into the body with the food or are formed in the body by chemical action. The significance of each may depend upon the amount which is present, as well as upon its mere presence or absence. In interpreting the meaning of each of the constituents of the urine its method of formation must be considered, as well as the factors which may cause this to vary.

Considerable variations are found in the composition of urine which has been collected at different times of the day. That which is passed immediately after rising may differ from that excreted an hour or two after the first meal both in the kind and amount of the dissolved solids. Sugar and albumin are more commonly excreted after a meal, and may be found then, yet not be present in the night's urine. In order to obtain a fair sample for testing, the urine should be collected for twenty-four hours and, after mixing, a part taken for analysis. In all quantitative determinations the volume for twenty-four hours must be measured, and when it has been determined how much of the substance is present in the portion tested, the amount contained in the whole day's urine should be calculated. A statement of the percent-

age alone has little value if the quantity of the urine is not taken into account. To avoid fermentation the vessels should be clean and the tests should be made as soon as possible. If it must be preserved, a thin layer of toluene on the surface is an effective preservative.

The average volume of the urine in twenty-four hours is, for an adult, between 900 c.c. and 1200 c.c. (30 and 40 ounces). This, however, is subject to great variations. It is increased by diuretics, by diseases, like diabetes and others; it is diminished in febrile diseases, in acute nephritis, in some other diseases of the kidneys, and usually before the fatal termination of a disease. Its variation gives indications of the progress of the disease. The volume will be also affected by the amount of drink or liquid food and, in general, varies inversely with the perspiration.

From the presence of ferments, the urine begins to undergo a change after it has stood a few hours. The reaction becomes alkaline, owing to the production of ammonium carbonate from the urea, and this precipitates some of the solids, so that the liquid loses its transparency. This and other decompositions produce disagreeable odors.

The odor of normal urine is characteristic. Certain foods and medicines change this; *e.g.*, oil of turpentine is said to give an odor of violets. In some cases the odor of the substance taken appears unchanged in the urine, but it is usually altered through the chemical changing of the agent. When it putrefies the odor is ammoniacal and offensive. In cystitis it is ammoniacal when passed. In suppurating diseases the odor may be putrid.

Fresh, normal urine is clear, but after standing a short time a cloud of mucus appears. Pathologically it may be

cloudy with matters which settle as a *sediment*. They will be discussed under that subject.

The color of urine is normally some shade of yellow, varying from nearly colorless to reddish yellow. The former is true of urines containing much water, and the latter where the urine is concentrated and of high specific gravity. The latter is constant in febrile conditions and their severity can here often be judged from the color. Pathologically the urine assumes many other shades. Presence of blood gives a red or, when methemoglobin is present, a brown. Jaundice gives a greenish cast or brownish green; melanotic cancer, almost black; typhus or cholera, sometimes blue, from indigo formed by decomposition. Some medicinal or poisonous substances change the color; thus senna or rhubarb gives a reddish or brownish color, which changes to blood red on adding an alkali. Santonin gives a yellow; carbolic acid and salol a dark green to black; antipyrin and quinine often darken it.

The specific gravity of urine varies with the amount of water and dissolved solids. With an increase of the water it approaches 1.000, and becomes greater as the solids increase. Hence it is easy to ascertain the amount of the solids which are present. If the second and third decimal figures of the specific gravity at 25° are multiplied by 2.6 it will give approximately the weight of dissolved solid substances in one thousand parts of urine (grams per liter) (Long's coefficient). Thus, urine of sp. gr. of 1.020 contains about 52 grams in a liter.

The specific gravity varies under normal conditions from 1.002 to 1.040. It is usually between 1.015 and 1.025. If sugar is not present the variation in specific gravity is due almost entirely to that of the urea. Clin-

ically the specific gravity of urine is determined by an hydrometer, called a urinometer, which consists of a spindle weighted so as to float in pure water at the line marked 1.000. The specific gravity is indicated by the figures on the spindle at the surface of the liquid. Urinometers should always be tested in pure water, and if they are not correct the reading in the urine must be changed to correspond with the error. Since the specific gravity varies with the temperature, some standard temperature must be adopted. Most instruments are graduated at 60° F. (15.6° C.). The urine must be brought to this temperature before testing or, if accuracy is desired, the reading corrected by adding 1 in the fourth decimal place for every degree Fahrenheit above 60° or subtracting 1 for each degree below 60°. (0.00018 for each degree centigrade above or below 15.6°). In order to obtain accurate results the degrees should not be too close together on the spindle.

The importance of a knowledge of the specific gravity is rather to detect marked changes in the urine from a series of observations than to be able to infer the presence of some abnormal constituent, like glucose, which would certainly be found by the subsequent tests. Thus, in nephritis a decrease in specific gravity without change in the volume indicates that the urea is not being excreted and that uremia may be feared.

468. Test the accuracy of the urinometer in water, then take the specific gravity of urine. The cylinder must be wide enough for the urinometer to float in it without touching. Foam on the liquid should be removed by a piece of filter paper.

469. Test with an accurate urinometer the difference in specific gravities of freshly passed urine when at a temperature of from 95° to 98° F. and that at 60° F. or below.

The reaction of normal mixed human urine passed during twenty-four hours is acid. Quantitative determinations of the salts in the urine show that the bases (cations) are not present in large enough amounts to replace all the hydrogen of the acids. This fact is commonly expressed in the statement that the acid reaction is due to acid salts, principally acid phosphates of sodium and potassium. However, since these as well as the other inorganic compounds are more or less dissociated, it is preferable to say that the hydrogen ion causes the acidity. The administration of alkaline drugs is followed by the urine's becoming less acid or even alkaline. The same effect is produced by vegetable foods. These contain the potassium salts of organic acids—citric, malic, tartaric, and others which are oxidized to potassium carbonate in the system. A similar result is brought about a short time after a hearty meal, when hydrochloric acid is being set free from its salts in the mucous cells of the stomach. The bases which are freed at the same time remain to increase the alkalinity of the blood. Part of them pass into the urine, producing the "alkaline tide," or alkaline reaction, which is often noticed at this time. The urine of herbivorous animals is normally alkaline from this cause. On the other hand, an acid food or one from which acids are produced during its decomposition in the body will increase the acidity. Such a one is lean meat, which contains sulphur and phosphorus compounds, which form sulphuric and phosphoric acids by oxidation. Hence the

reaction of the urine may be to a considerable extent regulated by the selection of foods.

Upon standing all urine becomes alkaline by fermentation. This is produced by the action of a number of micro-organisms upon the urea, resulting in the formation of ammonium carbonate:—



If these ferments are introduced into the bladder by an unclean catheter the same action is often produced there. In chronic inflammation of the urinary tract ammonium carbonate is usually present. The latter alkalinity—from ammonium carbonate—can be distinguished from that produced by sodium and potassium salts by the litmus paper resuming its red color after drying, if ammonia were the alkali, but not otherwise.

In determining the degree of acidity of the urine by the use of a standard alkaline solution, litmus cannot be used to indicate when the neutralization is complete, on account of the interference of the phosphates.

Excessive acidity of the urine causes, in time, an irritation of the urinary passages, and is favorable to the formation of uric acid concretions. Continued alkalinity makes a sediment in the urine, and tends to produce phosphatic calculi. It also produces irritation or inflammation of the mucous membrane.

470. Test the reaction of urine with sensitive litmus paper, and if alkaline determine whether it is caused by ammonium carbonate by the paper's turning red again after drying, or whether a sodium or potassium compound is the alkali by the paper's remaining blue on drying. If the urine is acid make portions of it alkaline by

each of the above compounds and try their reactions to litmus. Explain difference in results.

471. Determination of the Titratable Acidity of Urine.—Before titrating the calcium is precipitated by potassium oxalate. To 25 c.c. of urine in a small Erlenmeyer flask add 5 grams (about 5 c.c.) of powdered neutral potassium oxalate and a few drops of phenol-phthalein solution. Shake for 1 to 2 minutes and immediately titrate with 0.1 normal sodium hydroxide until a permanent pink color is obtained.

From 550 to 650 c.c. of 0.1 N alkali is usually required for normal urine excreted in twenty-four hours.

472. Collect the urine of the day in three-hour periods and determine if there is a variation in its acidity.

473. Take internally sodium acetate in 2 to 3 gram doses and note its effect upon the reaction of the urine. If alkaline, is it from ammonium carbonate or from the sodium or potassium salt?

474. Determination of Alkali Tolerance.—Where there is an abnormal amount of acid products formed in the body larger quantities of alkali are required to make the urine alkaline. The alkali tolerance of an individual is his ability to ingest an alkali without an alkaline excretion. It can be learned by administering sodium bicarbonate in small quantities at intervals and noting how much is taken before the urine becomes alkaline. Over 0.5 gram per kilogram of body weight indicates acidosis, and if it approaches 1.0 gram per kilogram the condition is serious.¹

¹ Consult Van Slyke. Journal of Biological Chemistry, 1918, xxxiii, 277.

Take 5 grams of sodium bicarbonate in 100 c.c. of water. At the end of half an hour pass urine and test it with litmus paper. Continue 5 gram doses of the bicarbonate each half hour, testing each time with litmus, until 0.5 gram per kilogram of body weight has been taken; unless the alkaline reaction appears previously. The test can be continued until the paper turns blue. A faint reaction may be made more visible by converting the bicarbonate into the normal carbonate through boiling.

475. The test should not be made after a heavy meal. Why?

476. **Determination of True Acidity of Urine** (Hydrogen ion concentration). Henderson and Palmer's Method.—This depends upon the use of colored indicators whose shade varies with the concentration of the hydrogen ion and the comparison of the color obtained with a sample of urine compared with that given by the same indicator in a solution whose acidity is known.

The indicators used are paranitrophenol, alizarine sodium sulphonate, phenol phthalein, neutral red and methyl red. The standard solutions of known acidity are prepared by mixing monosodium orthophosphate, NaH_2PO_4 , with disodium orthophosphate, Na_2HPO_4 , and acetic acid with sodium acetate. These solutions are made up of the normality represented in columns two and three of the following table. If equal volumes of these are mixed the figures in the fourth column are the logarithms of the hydrogen ion concentration, the minus sign being omitted (page 102). The brackets in the fifth column show the limits through which each indicator is best adapted for indicating the acidity.

TABLE A.

No.	NaH_2PO_4	Na_2HPO_4	P_{H^+}	Range of indicators
1		0.1000N	9.27	Phenol phthalein Neutral red
2	0.0001N	0.0480N	8.7	
3	0.0001N	0.0120N	8.0	
4	0.0166N	0.0833N	7.48	
5	0.0010N	0.0060N	7.38	
6	0.0010N	0.0023N	6.90	
	Acetic acid	Sodium acetate		Sodium alizarine sulphonate
7	0.0009N	0.0920N	6.70	
8	0.0023N	0.0920N	6.30	
9	0.0046N	0.0920N	6.00	
10	0.0092N	0.0920N	5.70	
11	0.0230N	0.0920N	5.30	
12	0.0460N	0.0920N	4.90	
13	0.0920N	0.0920N	4.70	Methyl red

Table B gives the actual concentration of the hydrogen ion corresponding to the minus logarithms. From this the acidity of the urine can be found.

TABLE B.

Log.	H^+	Log.	H^+	Log.	H^+
4.6	250×10^{-7}	5.8	16×10^{-7}	7.0	1.0×10^{-7}
4.8	160×10^{-7}	6.0	10×10^{-7}	7.2	0.63×10^{-7}
5.0	100×10^{-7}	6.2	6.3×10^{-7}	7.4	0.40×10^{-7}
5.2	63×10^{-7}	6.4	4.0×10^{-7}	7.6	0.25×10^{-7}
5.4	40×10^{-7}	6.6	2.5×10^{-7}	7.8	0.16×10^{-7}
5.6	25×10^{-7}	6.8	1.6×10^{-7}	8.0	0.10×10^{-7}

Prepare solutions of the indicators by dissolving:

Alizarine sodium sulphonate, 1 gram to 1000 c.c.; use 10 drops for each determination.

Methyl red, a saturated solution in 50 per cent. alcohol; use 3 to 4 drops for each determination.

Neutral red, 1 gram to 1000 c.c.; use 20 drops for each determination.

Paranitrophenol, 1 gram to 500 c.c.; use 15 drops for each determination.

Phenol phthalein, 1 gram to 500 c.c. of alcohol; use 3 to 5 drops.

In order to have the same shade in the urinary sample and the standard solutions it may be necessary to add to the colorless standards some coloring matters to imitate the color of the urine. For such a purpose are used paranitrophenol, alizarine sodium sulphonate, methyl orange and bismarck brown.

The determination is made in 250 c.c. flasks, indistinguishable in color and form, or in colorless glass test tubes about 160 mm. x 16 mm.

Prepare a standard series of 10 flasks by placing in each 10 c.c. of the successive mixtures of Table A; dilute to 250 c.c. with distilled water and add to each exactly the same volume of the sodium alizarine sulphonate solution—about 10 drops. Perform the same operation with 10 c.c. of urine in another flask and compare with the standard series to estimate the hydrogen ion concentration.

Then select from the other indicators the one adapted for the range of acidity indicated. Thus if the acidity is greater than 5.3 use methyl red; for 5.3 to 5.7 use paranitrophenol; for 6.9 to 8.0 use neutral red and for greater alkalinites, phenol phthalein, as follows:

I. With 0.15 c.c. of solution of methyl red use 10 c.c. of urine in a test tube, making up the standard series in the same manner.

II. With 15 drops of the paranitrophenol make the determination in flasks as with sodium alizarine sulphonate.

III. Use flasks similarly with 20 drops of neutral red.

IV. Use phenolphthalein with undiluted urine in test tubes.

In cases where the urine is highly colored the coloring matters listed above may be used to make the standards the same tint before adding the indicators.

477. The Determination of Ammonia in Urine (Folin and Bell's method¹).—The ammonia is separated from the urine through absorption by a synthetic silicate called Permutit. It displaces the sodium of this silicate, forming an insoluble compound which can be filtered out. From this compound the ammonia is set free by sodium hydroxide and its amount determined by Nesslerizing.

The most exact method of determining very small quantities of ammonia is by comparing the depth of color imparted by Nessler's reagent with that produced in a solution having known ammoniacal content.

Nessler's reagent.—Dissolve 75 grams of potassium iodide in 50 c.c. of water and stir in 100 grams of mercuric chloride. When it has dissolved dilute to 400 to 500 c.c. and filter if it is not perfectly clear, then dilute to 1000 c.c. Keep this for a stock solution. For use add to 300 c.c. of this potassium mercuric iodide solution 200 c.c. of 10 per cent. sodium hydroxide solution and 500 c.c. of water. With this ammonium compounds give a color ranging from a light yellow with such amounts as one in 10,000,000 to 100,000,000 parts of water to a darker yellow, or a precipitate with larger concentrations.

Place about 2 grams of the Permutit in a 200 c.c. volumetric flask; add about 5 c.c. of water (no more), and measure into it accurately from a pipette (Ostwald) 1 or 2 c.c. of urine. Rinse down the added urine with 1 to 5 c.c. of water and shake gently but continuously for five minutes. Rinse the powder to the bottom of the flask by 25 to 40 c.c. of water and decant. Repeat this washing by decantation once or twice more. Add a little

¹ Journal of Biological Chemistry, 1917, xxix, 329.

water to the powder, then 5 c.c. of 10 per cent, sodium hydroxide and, after mixing, fill about three-fourths full of ammonia-free, distilled water, shake and add 10 c.c. of Nessler's solution. Fill to the mark with water, mix and compare the color in a colorimeter with that produced by treating in the same manner a standard solution of ammonium sulphate containing 1 mg. of nitrogen in the form of ammonia in 1 c.c. (To make this dissolve, 4.716 grams of chemically pure ammonium sulphate in water and, after adding 5 grams of sulphuric acid to prevent mould, dilute to a liter.)

In the absence of a colorimeter compare the colors in Nessler tubes. These are flat bottomed tubes of colorless glass. Pour into one the colored liquid made by the action of the Nessler reagent on the solution from the Permutit. Imitate this shade by taking in another ammonia-free distilled water with a measured amount of the standard ammonium sulphate solution and Nessler's reagent. Calculate the amount of ammonia in the urine.

478. Determination of Degree of Acidosis from the Index of Acid Excretion in Urine (Fitz and Van Slyke¹).—When acid is formed in the body it is not usually eliminated in the free state, but is neutralized at the expense of basic substances in the blood or tissues, or, lacking these, by ammonia from interrupted nitrogen metabolism. To determine the degree of acidosis due to excess of acids over mineral bases, both the titratable acidity and the acids neutralized by ammonia must be known.

¹ Journal of Biological Chemistry, 1917, xxx, 389 and 1918, xxxiii, 271.

Use the mixed urine of the twenty-four hours, keeping it in a cool place to avoid fermentation. Determine the amount of ammonia by aeration and titration with N/10 acid. (Use 25 c.c. of urine with about a gram of dry sodium carbonate and some petroleum to prevent foaming in the apparatus employed for the same purpose in the determination of urea, Fig. 11, page 218.)

Draw a current of air through the urine, then through 20 c.c. of N/10 acid for an hour and a half. (Titrate with N/10 sodium hydroxide and Congo red for an indicator. Calculate the amount of ammonia.) Determine the amount of titratable acid in another portion with N/10 sodium hydroxide and phenol-phthalein (471). Find the body weight in kilograms.

The capacity of the plasma for carbon dioxide is very

$$\text{VC} \\ \text{nearly equal to } 80 - 5 \sqrt{\frac{VC}{W}}, \text{ in which}$$

80 is the maximum capacity, when titratable acid and ammonia approach zero.

V = 24-hour volume of urine in liters.

C = sum of ammonia (as c.c. of N/10 ammonia per liter) plus titratable acid (as c.c. of N/10 acid per liter of urine).

W = body weight in kilograms.

More than 27 c.c. for the sum of N/10 ammonia plus N/10 titratable acid indicates abnormal acidosis.¹

¹ Consult Van Slyke, Journal of Biological Chemistry, 1918, xxxiii, 277.

UREA.

About 86 per cent. of the nitrogen in the urine of a healthy man has been found to be in the urea, $\text{CO}(\text{NH}_2)_2$. Under pathological conditions, however, it may vary greatly from this. The absolute weight of urea varies between 20 and 40 grams daily, being somewhat less for a woman than for a man. In round numbers, we can say that it is about one ounce in twenty-four hours for the adult male.

Urea crystallizes in long, colorless, rhombic prisms. It is easily soluble in alcohol and in water; hence it never forms a sediment. It forms double compounds with acids, some of which, like the nitric and oxalic acid compounds, $\text{CO}(\text{NH}_2)_2 \cdot \text{HNO}_3$ and $[\text{CO}(\text{NH}_2)_2]_2 \cdot \text{H}_2\text{C}_2\text{O}_4$, are not easily soluble, and are used in separating the urea from urine. It forms similar insoluble compounds with many salts of the heavy metals, mercury, copper, etc.

When urea is brought into contact with a hypobromite or a hypochlorite, it is decomposed into carbon dioxide, nitrogen, and water:—



This decomposition is made use of to determine the amount of urea in urine by measuring the volume of the nitrogen set free. There are a great number of modifications in form of the apparatus employed,—Hüfner's, Doremus', Squibb's, and many others,—all based upon the same principle. They do not give absolutely accurate results, but are often sufficiently exact for clinical tests, and have the advantage of requiring but a short time for their execution. Where it is desirable to learn accurately

the amount of nitrogenous compounds excreted, it is best to find the total nitrogen by Kjeldahl's method. The solution of sodium hypobromite should be freshly prepared from bromine and sodium hydroxide, as it decomposes on standing. It contains a large excess of the sodium hydroxide which unites with the carbon dioxide.

Doremus' ureometer for determining the percentage of urea in urine consists of a short graduated tube closed

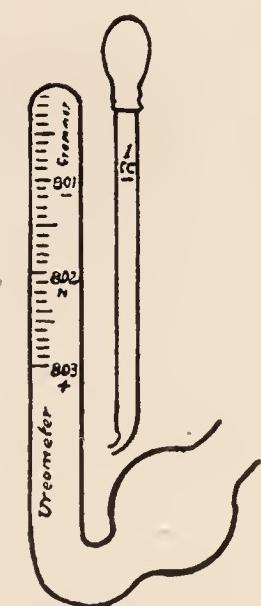


Fig. 10.—Doremus's ureometer.

at the upper end. Below it is bent upward and expands to a bulb. The graduations represent for each division 0.001 gram of urea. That is, 0.001 gram of urea evolves enough nitrogen to fill one division. Since one c.c. of urine is used, weighing very nearly a gram, nitrogen to fill one division corresponds very nearly to 0.1 per cent. of urea in the urine. With the tube is furnished a 1 c.c. dropping pipette.

Urea is formed chiefly in the liver, but also in the muscular tissues. Its source is the nitrogenous compounds

of the food and the tissues, including the blood, most of the nitrogen of such compounds being excreted from the body in the urea. Hence any increase in the destruction of these substances is accompanied by an increased formation of urea and *vice versa*. For this reason the urea is considered as a measure of the decomposition of the proteins in the body.

Some things which bring about an increased decomposition of proteins are: a large amount of nitrogenous food, like meat; very excessive exercise, which causes a destruction of tissue, though here the urea is not proportional to the exertion; fevers and inflammations up to the crisis, owing to the rapid loss of muscular tissue. After the crisis it is diminished. In phosphorus poisoning and diabetes mellitus the urea is excessive for the same reason. A greater excretion of water, either from excessive drinking or diuretics, carries with it a larger amount of urea, which seems to be thus washed out of the system.

On the other hand, less urea is excreted during hunger and sleep, when the metabolism of the body is lessened. Interference with the excretory power of the kidneys likewise diminishes the urea. This is seen in acute nephritis and other diseases of the kidneys. In such cases the production of urea is not stopped, but it accumulates in the system, often being accompanied by uremic poisoning. Since the urea is formed, at least in part, in the liver, we find that less is excreted in carcinoma and cirrhosis of this organ.

The fermentation of urea to ammonium carbonate, caused by the action of micro-organisms, has been already referred to.

Preparation of Urea.

479. From Urine.—(a) If only a small amount is desired, evaporate half a test tubeful of urine to dryness on the water bath. Dissolve the urea from the residue with 95 per cent. alcohol. Filter and evaporate the alcoholic filtrate to dryness, on a water bath. Dissolve the urea in a few drops of water; then allow crystallization to take place on a microscope slide. Examine the crystals under the microscope. If the form is not distinct, dissolve in a drop of water and again observe the crystals after this has evaporated. Add a drop of dilute nitric acid to the slide, let it stand a few minutes, then examine the crystals of urea nitrate. (Plate I, 6.) Sketch both.

(b) A larger quantity can best be obtained by evaporating half a liter to a liter of urine to a thin syrup upon the water bath, then cooling it in ice water, and adding about three times its volume of nitric acid of a specific gravity of 1.3 which has been boiled to expel the oxide of nitrogen and cooled with ice water. Filter off the urea nitrate through an asbestos or glass wool filter, washing with a small quantity of ice cold concentrated nitric acid. Dissolve the crystals in hot water and decolorize by chlorine water. Add, then, small portions of pure barium carbonate as long as it dissolves and until the liquid is neutral. Evaporate the whole upon the water bath to dryness. Pulverize the residue and dissolve the urea in absolute alcohol, which does not dissolve the barium nitrate. If the alcoholic solution is colored it can be decolorized by filtering it through bone black. Distill off the alcohol or allow it to evaporate to obtain the urea.

480. **Synthetically.**—Coarsely powder 25 grams of potassium ferrocyanide and heat in an iron dish over a Bunsen flame, stirring continually, until it has become a white powder and the lumps show no yellow color when they are broken. If it turns brown the heat is too high. Pulverize the mass as finely as possible in a mortar, mix it thoroughly with half its weight of finely powdered manganese dioxide, and heat in an iron dish under the hood, stirring meanwhile, until the mass glows and becomes thick and sticky. Heat until a small test dissolved in hydrochloric acid gives no blue color with ferric chloride. Then allow it to cool; dissolve the potassium cyanate, which has thus been formed with cold water. Convert this into ammonium cyanate by the addition of 19 grams of dry ammonium sulphate. Filter and evaporate upon the water bath at about 60° to 70°, at which temperature ammonium cyanate is converted into urea. The potassium sulphate crystallizes out first, and should be removed from time to time. At last evaporate to dryness and dissolve out the urea with absolute alcohol as before. Write equations for all reactions.

481. Mix a few of the dry crystals with soda-lime and heat in a dry test tube. The presence of nitrogen is shown by the evolution of ammonia.

482. Warm some crystals of pure urea in a dry test tube. It melts, then decomposes, yielding ammonia, which can be identified by litmus paper. When the substance has solidified, cool it, dissolve in water, make alkaline with sodium hydroxide, and add a few drops of copper sulphate solution. The color is due to the presence of biuret, $\text{NH}_2\text{CONHCONH}_2$. Write the equation for its formation.

483. To 5 c.c. of sodium hydroxide add a drop of bromine, and after this has dissolved a few crystals of urea. Explain the result of mixing the two reagents, also of the addition of urea. What is the gas?

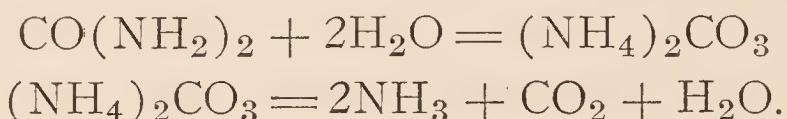
484. **Preparation of Sodium Hypobromite.**—In a thin glass flask or beaker containing 10 c.c. of water dissolve 4 grams of sodium hydroxide. Cool, and from a dropping pipette or funnel add slowly 1 c.c. of bromine, stirring or shaking meanwhile. Handle the bromine under a hood or in a draft of air to avoid the vapors, which are especially irritating to the eyes and lungs. As the bromine gas is heavy, it should be held below the level of the face while pouring, rather than above. Write equation.

485. Determine the percentage of urea in urine by the use of Doremus' ureometer (Fig. 10). First fill the tube with the hypobromite solution and invert it, having no more of the liquid in the bulb than is necessary to keep the tube full. Fill the pipette exactly to the mark with urine, insert the lower end into the ureometer, and slowly and steadily force the urine out by compressing the rubber bulb. The urine, being the lighter liquid, rises in the ureometer and the urea is immediately decomposed. The carbon dioxide is dissolved in the solution and only the nitrogen is collected. No gas bubbles should be allowed to escape into the ureometer bulb or back into the pipette, thereby causing a loss. When the foam has disappeared, read off the quantity of gas and calculate the percentage of urea. Duplicate tests should not differ more than 0.1 per cent. Hypobromite solution of this concentration can be used for several successive determinations. If the volume of urine in twenty-four

hours is known, calculate the weight of urea excreted in that time. If it is not known, assume it to be one liter. Write equation for decomposition of urea.

486. The Quantitative Determination of Urea (Urease Method).—This is probably the best method of determining the amount of urea in body fluids like urine or blood.

Urease is a urea-splitting ferment which occurs in various plants, particularly in the soy bean and the jack bean. By its action urea is hydrolyzed, ammonium carbonate being formed, which readily gives up its ammonia.



The amount of ammonia can be determined by Nesslerizing the solution or by titrating it with a standard acid. For more accurate determinations the ammonia should be blown out by a current of air into acid, where it can be titrated or Nesslerized. There are several modifications of this process. Three are given below.

487. Marshall's Method.¹—Measure with a pipette two 5 c.c. portions of urine into two 200 to 300 c.c. flasks and dilute to 100 to 125 c.c. Add to each a few drops of toluene to prevent bacterial action, and to one 2 c.c. of the neutral urease solution. This urease solution is made by letting 25 grams of finely ground soy bean meal stand for an hour in 250 c.c. of water with occasional shaking; then add 25 c.c. of decinormal hydrochloric acid and, after a few minutes at 35°, filter, and preserve the filtrate with a few drops of toluene. It should then be slightly alkaline to methyl orange; if it is acid it is not

¹ Journal of Biological Chemistry, 1913, xiv, 483.

very active. (This solution retains its activity as much as five days.)

Let the two flasks stand, tightly stoppered, over night at room temperature, then titrate each with decinormal hydrochloric acid, using methyl orange for an indicator. The alkalinity of the one without urease is due to the original constituents of the urine, that of the one with urease flask to the natural urinary alkalinity plus that of the ammonium carbonate formed from the urea. Since 1 c.c. of decinormal acid corresponds to 3 mg. of urea, the weight of urea in grams per liter is found by multiplying the number of cubic centimeters of decinormal acid used by 0.6.

488. Van Slyke and Cullen's Method.¹—In this a purified urease solution is used and the ammonia is blown from the fermented liquid into a standard acid. To prepare the purified urease, digest at room temperature for an hour one part by weight of soy bean meal with five parts of water. Filter through wood pulp, or centrifuge the mixture. Pour the solution into ten times its volume of acetone, which dehydrates the urease. Filter and dry the precipitate in a vacuum desiccator; it keeps indefinitely. For use dissolve one part in ten parts of water.

Measure into the cylinder *A* (Fig. 11) exactly 0.5 c.c. of urine with an Ostwald pipette; 5.0 c.c. of 0.6 per cent. NaH_2PO_4 or KH_2PO_4 neutralizing solution; 1.0 c.c. of the 10 per cent. urease solution and two drops of caprylic acid to prevent foaming. (Kerosene can be used in absence of the latter, though it is not as effective. A mixture of two volumes of amyl alcohol and three vol-

¹ Journal of Biological Chemistry, 1914, xix, 214.

umes of diphenyloxide is cheap and efficient.) Close with a stopper and let it stand for fifteen minutes.

Measure into the second cylinder, *B*, 25 c.c. of $\frac{N}{50}$ hydrochloric or sulphuric acid, a drop of 1 per cent. sod-

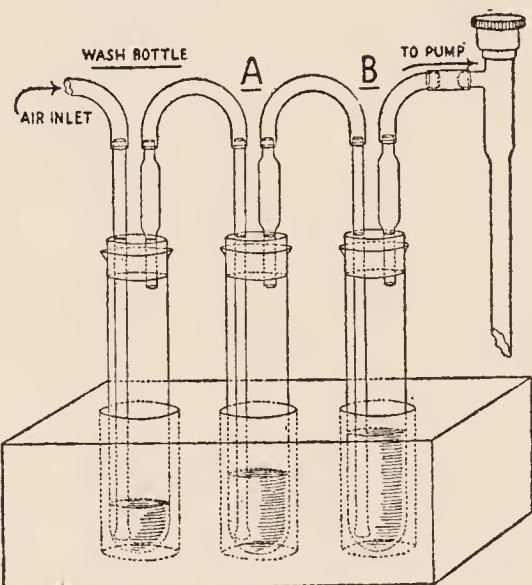


Fig. 11.—Apparatus for collecting ammonia from urine. Tube *A* contains the urine with urease, when the urea is to be decomposed. Tube *B* contains the standard acid into which the ammonia from *A* is swept by the current of air drawn by the pump. The first tube contains dilute sulphuric acid to remove any ammonia which may be in the air.

ium alizarine sulphonate and a drop of caprylic acid or other preventer of foaming. After fifteen minutes slowly draw the air through the fermentation tube into the acid for a minute, then remove the stopper of *A*, add an equal volume of saturated potassium carbonate and aerate again, slowly at first, then moderately rapidly for five to ten minutes. Titrate the remaining acid in *B* with fiftieth normal sodium hydroxide. The difference between this and the 25 c.c. taken represents the ammonia of the urine plus that formed from the urea. To get the former, perform the operation without urease, but using 5 c.c. of urine with the potassium carbonate.

$$0.56 \times \text{c.c.} \frac{\text{N}}{50} \text{ acid} = \text{grams of nitrogen.}$$

Weight of nitrogen \times 2.14 = amount of urea in 0.5 c.c. urine. The ammonia nitrogen and urea nitrogen can be determined at the same time by using an extra pair of tubes and aerating both sets at once.

It should be noted that standard alkali solutions, especially the very weak ones, like N/50 NaOH, will dissolve alkali from glass. They must be kept in paraffin lined bottles or be freshly prepared.

489. Folin and Denis' Method.—In this the ammonia from the ammonium carbonate formed from the urea by urease fermentation is determined by Nesslerization.

Measure 1 c.c. of urine into a 100 c.c. volumetric flask by means of an accurate (Ostwald) pipette and add 0.10 to 0.25 gram of soy bean meal in form of a 1 per cent. suspension. (Make the soy bean suspension by rubbing 5 grams of the meal with 15 c.c. of water to a paste in a mortar; mix with more water and rinse into a flask, using about 400 c.c. of water. Add 100 c.c. of alcohol, making about 500 c.c. Use 10 to 15 c.c. for each determination. It remains good at least two days.) Stopper the flask and let it stand for an hour at room temperature, or for fifteen minutes in a water bath at 50°. Add 25 c.c. of water and 1 c.c. of freshly prepared metaphosphoric acid solution. Mix and add one gram of blood charcoal and about 25 c.c. more of water. Shake, dilute to the mark and filter through a dry filter.

Measure with a pipette a definite amount of the filtrate into a 100 c.c. measuring flask. (The amount taken should contain from 0.7 to 1.3 mg. of nitrogen in the form of ammonia: for this usually from 5 c.c. to 20 c.c.

is the proper amount.) Dilute to 60 to 70 c.c. with ammonia-free, distilled water, add 15 c.c. of Nessler's reagent and compare the color in a colorimeter with that from a standard solution of ammonium sulphate containing 1 mg. of ammonia nitrogen in 1 c.c. (To make this, dissolve 4.716 grams of pure ammonium sulphate in water and dilute to a liter after adding enough sulphuric acid to make it 0.2 normal. This prevents moulds.)

In absence of a colorimeter compare the two colors in two Nessler tubes. When they are the same the volume of the standard solution used indicates the amount of urea nitrogen present. Multiplying this by 2.14 gives the weight of urea in the volume of urine taken. Calculate the amount per day (if the volume of urine is unknown, assume it to be 1000 c.c.).

490. Determination of the Total Nitrogen in Urine (Kjeldahl's method¹).—I. Prepare the following solutions:—

1. Standard sulphuric acid containing about 25 grams per liter, of which the strength has been accurately determined.
2. Standard ammonia, of which three to five volumes are necessary to neutralize one of the acid. Determine this accurately and calculate the amount of ammonia by weight in 1 c.c.
3. Sodium hydroxide free from ammonia and nitric acid, about 270 grams per liter.
4. Congo red, of which the solution contains 0.2 gram in 100 c.c. This is turned red by alkalies and blue by acids—the opposite of litmus.

¹ This method can be used for finding the amount of N in most animal and vegetable compounds.

Have also at hand:—

1. Sulphuric acid, sp. gr. 1.84, free from compounds of nitrogen.
2. Yellow mercuric oxide.
3. Powdered potassium permanganate.
4. Crystallized sodium thiosulphate.

II. *Operation.*—With a pipette measure accurately 5 c.c. of urine. Place it in a flask holding about 700 c.c., best of Jena glass. Add 0.4 gram of mercuric oxide and 10 c.c. of the *concentrated* sulphuric acid. Lay the flask in a slanting position on a wire gauze over a flame small enough to just bring it to boiling. Perform this operation under a hood or where there is a good draft to carry away the fumes of the acid. Continue the heating until the liquid is colorless or straw colored, which may require from thirty minutes to an hour. Then remove the flask from the flame and very slowly add to it a small amount of the powdered permanganate until it is colored reddish or greenish. The organic matter has been oxidized in this process, the nitrogen being converted into ammonia, which is contained in solution as ammonium sulphate.

The ammonia, after cooling and diluting to about 200 c.c., is to be set free by sodium hydroxide, then distilled into a known amount of standard acid, and its amount found by ascertaining the loss in concentration of the acid through its neutralization by the ammonia. For this purpose a condenser is to be arranged (Fig. 12) so that the flask can be connected with the upper end by means of a bent tube; this should be at least $\frac{1}{4}$ inch in diameter inside and a foot long, to prevent small drops of the boiling liquid's being carried over. The insertion of a bulb between the flask and condenser, also having the lower

end of the bent tube, in the flask, cut off obliquely, will aid in preventing this. Add, now, to the liquid in the flask about a gram of sodium thiosulphate to precipitate the mercury, and 80 c.c. of the sodium hydroxide, or enough to make it alkaline. Connect immediately with the condenser and distill into a 400 or 500 c.c. conical

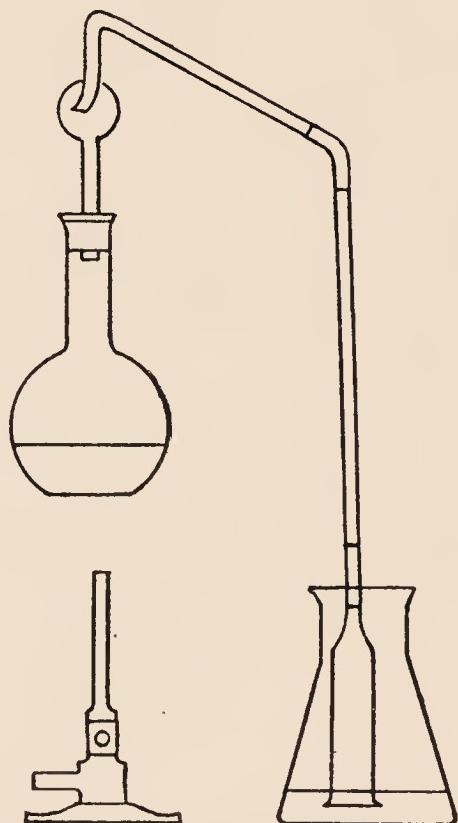


Fig. 12.—Apparatus for distilling ammonia into standard acid. The distillation flask is provided with a safety bulb to prevent spattering over of the sodium hydroxide. At the end of the tube a wide adapter is attached by rubber tubing to prevent back suction of the acid into the flask if boiling is interrupted. This adapter must dip slightly below the surface of the standard acid.

If convenient a condenser, such as Liebig's, can be substituted for the long tube but the end must dip into the acid to avoid loss of ammonia.

flask (Erlenmeyer) which contains exactly 10 c.c. of the *standard* acid (not the concentrated). A wide adapter tube attached to the lower end of the condenser should dip into the acid. Continue the distillation until at least

half has been distilled over and the distillate coming from the condenser no longer turns red litmus paper blue.

Then find how much ammonia has been taken up by the standard acid. To do this add a few drops of Congo red solution to the distillate. It will be colored blue, because of the acid reaction. From a burette add the standard ammonia, stirring meanwhile, until the blue just changes to a pink, when the liquid is neutral. Subtract the number of cubic centimeters of ammonia used from the number which are required to neutralize 10 c.c. of the standard acid. The difference represents the volume of standard ammonia equal to that which was distilled from the oxidized urine. Calculate the weight of NH_3 in this. Fourteen-seventeenth of the NH_3 is the weight of the nitrogen in 5 c.c. of urine. Calculate the percentage.

491. Equations for the liberation of ammonia gas, for the action of the gas upon the standard acid, for the titration of the residual acid.

492. **The Gunning Modification of the Kjeldahl Method for Nitrogen.**—No mercuric oxide, potassium permanganate or sodium thiosulphate are used but instead sodium sulphate is added to the sulphuric acid in digesting.

Mix the substance with the concentrated sulphuric acid as in the Kjeldahl method, add 10 grams (about 10 c.c.) of crystallized sodium sulphate and digest until the mixture is entirely colorless; dilute, make alkaline, distill and titrate as before.

493. **Colorimetric Micromethod for Determination of Urinary Nitrogen (Folin and Denis).¹**—In this a very small quan-

¹ Journal of Biological Chemistry, 1916, xxvi, 473.

tity of urine is employed, the nitrogen being converted to ammonia by heating with acid, similarly to the Kjeldahl method, then the ammonia is determined by Nessler's reagent.

Beside Nessler's reagent there are used a solution of 0.232 gram of ammonium sulphate per liter, 20 c.c. of which contains 1 milligram of nitrogen, and an acid oxidizing mixture containing 100 c.c. of concentrated sulphuric acid, 300 c.c. of 85 per cent. orthophosphoric acid and 15 c.c. of 10 per cent. crystallized copper sulphate solution.

Dilute the urine so that 1 c.c. contains from 0.7 to 1.5 mg. of nitrogen. That is, urine with a specific gravity of 1.018 or less should be diluted five times, of 1.030 or over twenty times, between these, ten times. With an accurate pipette (Ostwald) measure 1 c.c. of this diluted urine into a test tube about 190 mm. x 15 mm. Add 1 c.c. of the acid mixture and a small pebble or piece of pumice to prevent bumping and heat over a microburner, holding the bottom of the tube within 1 cm. of the burner until the water has been driven off and the sulphuric acid fumes appear. This should require 2 to 5 minutes. Then cover the mouth of the tube with a small watch glass and heat 0.5 to 3 minutes when the liquid should be clear yellow, blue or green. Heat then half a minute to a minute and let it cool two minutes. Add water and rinse into a 200 c.c. volumetric flask with about 150 c.c. of water.

This acid solution is to be neutralized with 10 per cent. sodium hydroxide. Find out how much of this hydroxide is required to neutralize 1 c.c. of the acid mixture by running it in from a burette with phenol phthalein for an indicator; then add $1\frac{1}{8}$ of this amount to the flask with 2 c.c. additional.

For a standard of comparison introduce 20 c.c. of the ammonium sulphate solution into another similar volumetric flask and treat with 1 c.c. of the acid mixture and as much of the sodium hydroxide as was employed before.

Add to each flask 15 c.c. of the Nessler reagent, mix and fill to the mark. Mix thoroughly. Filter a portion through a cotton plug or centrifuge it. Set the Duboscq colorimeter at 20 with the standard in both cups until the fields are alike in color. Then replace one with the unknown and by comparison determine its content of ammonia. Repeat the reading several times for accuracy.

494. The Determination of Urinary Ammonia.—After freeing the ammonia from its compounds by an alkali it is carried out by an air current and the amount is found after absorption in an acid of known concentration.

For the liberation of ammonia and the absorption in the standard acid use glass cylinders 4 to 5 centimeters in diameter and 20 to 25 centimeters in height each with an inlet tube passing nearly to the bottom and an outlet tube at or near the top (Fig. 11). It is well to have at the bottom of the inlet tube a number of small holes instead of one large one in order to better divide the current of air.

To 25 c.c. of urine in one tube add 16 grams of sodium chloride and 0.5 to 1.0 gram of sodium hydroxide, covering the liquid with a thin layer of kerosene to prevent foaming. (A drop of caprylic acid or phenyl ether is better.¹) Connect the outlet tube of this cylinder with the inlet tube of the other in which is 10 c.c. of standard sulphuric acid of the same concentration as that used in Kjeldahl's method for determining nitrogen (490). Connect the outlet tube of the standard acid cylinder with a Bunsen pump and draw through both cylinders a rapid current of air for an hour, or until all ammonia has been carried out.

Titrate the sulphuric acid after this using ammonia and an indicator exactly as in the Kjeldahl determination and calculate the weight of ammonia absorbed by the acid.

The amino acids produced in the intestine by the erepsin cleavage of peptones are one stage in the transformation of proteins to urea. They are absorbed and carried by the blood to the liver and other organs. There part of them may be synthesized into proteins again. Part of them are deaminized, that is, the amino group is split off as ammonia.



The ammonia is converted into salts by the metabolic acid products of the blood and tissues. Those like ammonium carbonate $(\text{NH}_4)_2\text{CO}_3$, and ammonium carbamate, $\text{NH}_4\text{CO}_3\text{NH}_2$, are transformed into urea, as can be shown by transfusing them through a liver, or by leaving them for a time in contact with

¹ Journal of Biological Chemistry, 1918, xxxiii, 373.

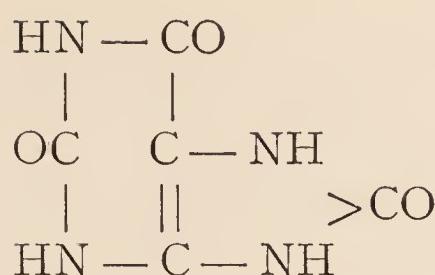
pulped liver. The presence of acids in the system (abnormal in either kind or amount) converts the ammonia into stable salts and thus prevents this change into urea. Normally, then, both ammonia and amino acids are intermediate metabolic products and should not be found abundantly in the urine.

495. The Quantitative Determination of Ammonia and Amino Acids (Formaldehyde Method).—This depends upon the fact that ammonia which is in combination with acids in the form of salts will, if formaldehyde (formalin) is added, leave the acids to unite with the formaldehyde with a production of hexamethylenetetramin. The amount of the acids thus set free can be determined by titration with a standard alkali and the ammonia calculated. Amino acids give a similar reaction.

Dilute 20 c.c. of urine three to five times with water, and add a few crystals of neutral potassium oxalate and a few drops of an alcoholic solution of phenolphthalein. From a burette run in slowly, while stirring, a decinormal solution of sodium hydroxide (4 grams in 1 liter) until a permanent pink color appears. Then add 2 c.c. of a 40 per cent. solution of formaldehyde which has been previously neutralized. When the pink color has disappeared pass in more of the decinormal sodium hydroxide until the color returns. The alkali used in this second titration corresponds to the amount of ammonia plus amino acids present. To distinguish them aspirate the ammonia into acid as in 487, neutralize carefully and make the formaldehyde titration in this and the aspirated portion separately. Each c.c. used represents 0.0017 gram of ammonia, NH_3 , in 20 c.c. of urine. Knowing the volume of urine for twenty-four hours, the total daily ammonia can be calculated.

URIC ACID.

Uric acid is normally present in solution in the tissues, blood, and urine of mammals. With birds and snakes it is the principal nitrogenous excretory product. Its formula is $\text{C}_5\text{H}_4\text{N}_4\text{O}_3$ and the constitution of the molecule is probably



It is consequently 2-6-8-trioxypurin.

The daily amount varies much, but averages from 0.2 to 0.8 gram. It is formed from the catabolism of the nuclein compounds of the foods and body tissues, that from the former being termed exogenous, and from the latter, endogenous uric acid.

Uric acid is comparatively insoluble in water or acids, but dissolves readily in the fixed alkalies, forming salts of uric acid, or urates. In the urine the acid exists in the form of these salts or united with some organic base. It is a dibasic acid, having two atoms of hydrogen which can be replaced by metals. It can thus have two series of salts, the acid and the normal, corresponding to HKSO_4 and $\text{K}_2\text{S}\text{O}_4$. Of these classes the normal salts of the alkalies are quite soluble in water, but the acid salts do not dissolve so easily. The acid can be set free from its salts by the use of a stronger acid. It is soluble in cold concentrated sulphuric acid and precipitates when this is diluted. The solubility of the acid salts is much less in cold water than in warm. Consequently they frequently separate from urine which was clear when passed but has stood in a cold room, and they can then be redissolved by warming.

When it is pure, uric acid exists in the form of colorless crystals. As it is found in the urine, it, as well as its salts, is always colored yellow to brown by the coloring matter which has been carried down from the urine. The simplest form of crystals is tabular with

curved sides and pointed ends. These are frequently united at right angles, making a star-shaped form, two of the rays often being smaller than the other two. In urinary sediments many crystals may be united, making a rosette-like form. In strongly acid urine the crystals sometimes have jagged edges like the teeth of a broken comb. Many different forms may be obtained by precipitating with various concentrations of acid. (Plate II, 11.)

Uric acid and its salts have, in some degree, the power of reducing copper compounds in an alkaline solution and thus give with Fehling's or Trommer's or other reduction tests results which are similar to those obtained with glucose. When the dry substance is warmed with nitric acid it is oxidized, and then gives with ammonia a reddish purple salt, which serves to detect and identify the acid.

The urates as found in the urine are either in solution or form a sediment. The latter is generally amorphous and is always colored yellow to brown. Acid sodium urate may occur in spherical aggregations of microscopic acicular crystals. Ammonium urate, formed when urine becomes alkaline by fermentation, may be found as brownish spherules covered with irregular spicules, the so-called "thorn apple" crystals. (Plate II, 9 and 11.) The amount of uric acid in urine is sometimes found by precipitating from a measured volume of urine by hydrochloric acid, the albumin having first been removed if it is present. After washing the crystals they are weighed. The results thus obtained are too low, because of the slight solubility of the crystals in water. Volumetric or

colorimetric methods may also be employed. These are more accurate and no more difficult.

496. Prepare uric acid from urine by adding to a beakerful 1 to 2 per cent. of concentrated hydrochloric acid. In twenty-four hours the uric acid will have separated. Examine the crystals under the microscope. Decant off the water and dissolve some of the crystals in 2 to 3 c.c. of concentrated sulphuric acid. Precipitate by gradually adding 10 to 20 times as much water. Compare the color and shape of these purified crystals with corresponding properties of the original crystals.

497. Precipitate from urine the uric acid with acids of varying concentration, acting for different times. Sketch the principal forms obtained.

498. Dissolve a few of the crystals of the acid in a little sodium hydroxide and add a few drops of Fehling's solution. Boil and the red cuprous oxide will be formed, best seen by the use of a dark background.

499. To a small quantity of uric acid in a porcelain dish add a few drops of dilute nitric acid and evaporate to dryness, holding the dish over a small flame with the hand in order to avoid heating too highly. A reddish yellow residue is left. Pour into the dish a drop of ammonia without at first letting it come directly into contact with the residue. In a short time the residue becomes colored reddish purple. The ammonia may be added directly to the residue if an excess is not used. An excess destroys the color. The addition of a drop of sodium hydroxide changes the color to a bluish purple, which is destroyed on warming. This is called the *murexide reaction*.

500. **Preparation of Amorphous Acid Urates.**—Dissolve uric acid in a slight excess of sodium hydroxide, and then pass carbon dioxide into the cold solution until it is saturated. Acid sodium urate separates in amorphous masses. Write two equations for solution and precipitation.

501. Test the solubility of the acid sodium urate by warming with a small quantity of water. It will dissolve, and, if not too much water has been used, will separate out again when it cools.

502. Prepare crystallized acid urates by dissolving a little uric acid in a warm solution of sodium phosphate. Filter, if necessary, and allow the filtrate to stand and evaporate. The sodium urate will crystallize as masses of acicular crystals. Sketch them. What is their color?

503. **Volumetric Determination of Uric Acid (Folin-Shaffer method).**—Prepare a solution in water, one liter of which shall contain 500 grams of ammonium sulphate, 5 grams of uranium acetate and 6 c.c. of glacial acetic acid. Of this add 75 c.c. to 300 c.c. of urine in a 500 c.c. flask, mix, and after five minutes filter through a plaited filter. Take two portions of the filtrate, 125 c.c. each, pour them into beakers, add 5 c.c. of concentrated ammonia and let the precipitated urates stand until the next day. Filter and wash the urates, using a 10 per cent. ammonium sulphate solution for this as well as for transferring to the filter. Then spread out the paper and wash off the precipitates into beakers, using about 100 c.c. of water for each. Add 15 c.c. of concentrated sulphuric acid and titrate immediately with one-twentieth normal potassium permanganate, containing 1.57 grams per liter, stopping when the solution is first pink throughout. For each cubic centimeter of permanganate 0.00375 gram of uric acid has been oxidized. Calculate the amount in the urine,

adding a correction of 0.003 gram for every 100 c.c. of urine employed because of the solubility of the urates.

504. Colorimetric Method for Determination of Uric Acid (Folin and Denis, modified by Benedict and Hitchcock).—In this the uric acid is precipitated as the silver salt which is washed clean and dissolved in potassium cyanide. This solution is then treated with phosphotungstic acid which it reduces to a deep blue compound. The amount of uric acid is estimated by comparing in a colorimeter this color with that obtained in the same manner from a known weight of uric acid. The solutions needed are:

Standard uric acid. Dissolve in 200 to 300 c.c. of hot water 9 grams of pure crystallized disodium hydrogen phosphate, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and 1 gram of crystallized sodium dihydrogen phosphate, $\text{NaH}_2\text{PO}_4 \cdot 4\text{H}_2\text{O}$, filter if not clear, dilute with hot water to about 500 c.c. and pour upon exactly 200 mg. of pure uric acid suspended in a little water in a liter flask. Shake until the acid dissolves, cool, add exactly 1.4 c.c. of glacial acetic acid, dilute to the mark and mix. Add 5 c.c. of chloroform to prevent growth of moulds and bacteria. Five c.c. contain 1 mg. of uric acid.

Ammoniacal silver solution; this contains

Magnesia mixture ¹	30 c.c.
Three per cent. silver lactate solution	70 c.c.
Concentrated aqueous ammonia	100 c.c.

Phosphotungstic acid solution (uric acid reagent). Boil together 100 grams of sodium tungstate, 80 c.c. of 85 per cent. phosphoric acid and 750 c.c. of water in a flask with a reflux condenser for an hour and a half; cool and dilute to 1 liter.

Potassium cyanide; a 5 per cent. aqueous solution.

Such a volume of urine as will contain from 0.7 to 1.3 mg. of uric acid (2 to 4 c.c. is usually the right amount) is accurately measured into a centrifuge tube, diluted with water to about 5

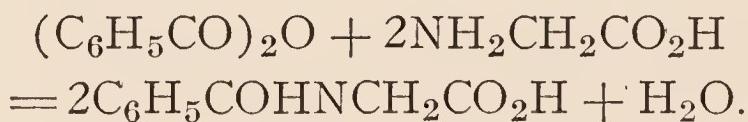
¹ To make magnesia mixture dissolve 17.5 grams of crystallized magnesium sulphate and 35 grams of ammonium chloride in about 100 c.c. of water; add 60 c.c. of concentrated aqueous ammonia and dilute to 200 c.c.

c.c. and treated with 15 to 20 drops of the ammoniacal silver solution. Mix the liquid with a small stirring rod and centrifuge for 1 to 2 minutes. Pour off the supernatant liquid as completely as possible, the tube being finally inverted and the inside of the lip touched with a piece of filter paper to remove the last drop. Treat the residue in the tube with 2 drops of the 5 per cent. cyanide solution and stir thoroughly for half a minute with a narrow stirring rod. Add 0.5 c.c. to 1.0 c.c. of water and stir again. Add 2 c.c. of the phosphotungstic acid solution, pour in 10 c.c. of a 20 per cent. solution of sodium carbonate, and at the end of half a minute wash it quantitatively into a 50 c.c. volumetric flask and dilute to the mark.

Treat 5 c.c. of the standard uric acid solution in exactly the same way at the same time, then compare the colors of the two in a colorimeter, calculating the weight of uric acid from the urine by this comparison.

HIPPURIC ACID ($C_6H_5COHNCH_2CO_2H$).

This occurs normally in the urine, the average for a man being about 0.7 gram per day. It is found here in larger amounts after the internal use of benzoic acid. It increases with a vegetable diet, such as cranberries and plums, and is abundant in the urine of herbivorous animals. Some is formed by intestinal putrefaction. It forms translucent, four-sided prisms, somewhat soluble in water. The acid can be made synthetically by heating benzoic anhydride (C_6H_5CO)₂O, with glycocoll, $NH_2CH_2CO_2H$:-



When hippuric acid is heated with mineral acids or alkalies it decomposes again into glycocoll and benzoic acid.

505. Preparation of Hippuric Acid.—Take internally 2 grams of pure sodium benzoate and collect the urine

for the next twenty-four hours. Make it strongly alkaline with milk of lime. Warm, filter, and evaporate the filtrate to a syrup on the water bath. After it has cooled acidify strongly with concentrated hydrochloric acid. Stir and filter, washing with a little very cold water. Dissolve the crystals in the smallest possible amount of boiling water. To destroy the coloring matter pass chlorine gas into the hot solution until it is light yellow. Then cool it, filter, and wash the crystals with a very little cold water. If they are still colored they can be still further purified by dissolving in water and boiling with a little animal charcoal. Filter, and let the acid crystallize from the filtrate. Sketch the crystals.

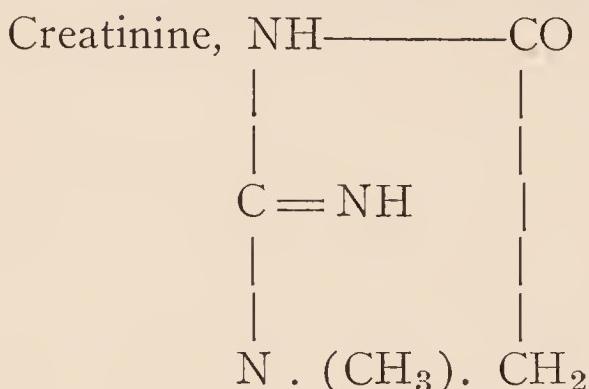
506. Heat a few of the dry crystals in a glass tube. They melt and turn red, then give, at first, a hay like odor, afterward the odor of bitter almonds, from the hydrocyanic acid formed. On the cooler part of the tube is a sublimate of benzoic acid.

507. On a few crystals in a test tube pour about a cubic centimeter of concentrated nitric acid, and bring to a boil. Evaporate to dryness in a porcelain dish on a water bath. The residue, when heated in a dry glass tube, gives the odor of bitter almonds (nitrobenzene). This test can be used to detect small quantities of hippuric acid.

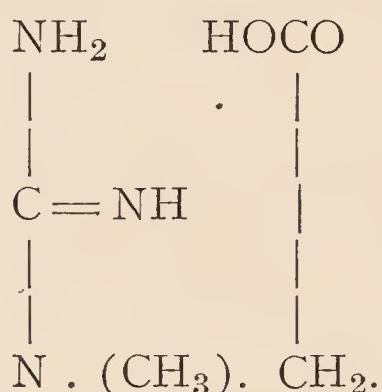
508. **Preparation of Glycocol from Hippuric Acid.**—Boil in a flask 1 part of the acid for ten to twelve hours with 4 parts of dilute sulphuric acid. Use an inverted condenser to prevent evaporation. Let the liquid cool and filter out the benzoic acid. Concentrate the filtrate and mix it in a separatory funnel with ether, which removes the last traces of benzoic acid. Separate the aqueous solution of glycocol from the ether, add to it barium carbonate until the sulphuric acid has been neutralized,

filter, wash and evaporate the filtrate until the glycocoll commences to crystallize. It has a sweet taste, whence its name.

CREATININE AND CREATINE.



is the anhydride of creatine,



Creatine is found in the blood and muscular tissue, but not normally in the urine of adults. Boiling with acids or alkalies converts it to creatinine.

Creatinine is always present in the urine and has its origin largely in the muscular system. It varies in the urine with the basic muscular metabolism and is practically a constant amount for any given individual. It increases in the urine with foods, such as meat, which contain it or its precursors, and varies with diseases which affect muscular metabolism.

509. Add to 5 c.c. of urine a few drops of a freshly prepared solution of sodium nitroprusside, then make it alkaline with sodium hydroxide. The liquid becomes

red, slowly turning to yellow (Weyl's test). While red, acidify strongly with acetic acid. It immediately turns yellow. Compare with Legal's test for acetone (558).

Acidify the yellow solution from Weyl's test with acetic acid and heat. A green, followed by a yellow color appears.

510. Add picric acid to urine, then make it alkaline with sodium hydroxide. It turns red (Jaffé's test). The color very slowly disappears.

511. **The Preparation of Pure Creatinine from Urine (Folin-Benedict method).**—Dissolve 18 grams of picric acid in 45 c.c. of alcohol by boiling and while hot pour it into a liter of fresh urine in a large beaker. Let it stand over night, decant off the liquid and filter with a Buchner funnel with suction, washing once or twice with cold saturated picric acid solution. Rub this mass from the suction filter in a mortar for 3 to 5 minutes with enough concentrated hydrochloric acid to form a thin paste then filter with suction on a hardened paper washing twice with enough water to cover the residue and sucking as dry as possible. Transfer the precipitate to a flask and add small portions of dry magnesium oxide to neutralize it, keeping the flask cool by running water. Filter out the creatinine solution from the residue by suction and immediately add enough glacial acetic acid to the filtrate to make it strongly acid. Dilute this with about four volumes of 95 per cent. alcohol and filter out the slight precipitate after 15 minutes. Mix the filtrate with 5 c.c. of 30 per cent. zinc chloride solution and let it stand over night in a cool place. Filter the creatinine-zinc chloride with a Buchner funnel, wash once with water, then with 50 per cent. alcohol, finally with 95 per cent. alcohol. The yield should be 1.5 to 1.8 gram per liter of urine.

To purify the compound dissolve it, using for each gram 10 c.c. of water and 6 c.c. of normal sulphuric acid, heating until it is clear. Add a little purified animal charcoal, boil a minute and filter pouring the filtrate back several times if it is not colorless at first. Wash with a little hot water and to the hot

solution add about 1 c.c. of the concentrated zinc chloride solution and 1 gram of potassium acetate dissolved in a little water. After about ten minutes add an equal volume of alcohol and let it stand in a cool place. Examine the crystals under the microscope. Traces of potassium sulphate remaining may be removed by recrystallizing from a little water and washing with alcohol.

To obtain the creatinine from its zinc compound place it in a flask and add several times its weight of concentrated ammonium hydroxide and warm gently until it dissolves. Close tightly and set in the ice-box until the creatinine crystallizes. Examine the crystals and preserve for making standard solutions.

512. The Determination of Creatinine (Folin's Method).—This depends upon a comparison of the color obtained by picric acid, in an alkaline solution, with that of a standard solution of potassium bichromate.

To 10 c.c. of urine in a 500 c.c. measuring flask add 15 c.c. of a saturated solution of picric acid and 5 c.c. of a 10 per cent. solution of sodium hydroxide, letting the mixture stand five minutes; then dilute to the mark with water and mix thoroughly. Fill one tube of a Duboscq colorimeter to a depth of 40 mm. with the standard potassium dichromate solution and the other tube with the creatinine solution. Turn the screw to give the same shade of reddish yellow. Take the average readings of three tests, which should not differ more than 0.3 mm. from each other. The number of milligrams of creatinine in the 10 c.c. of urine used is found by dividing 81 by the number representing the height of the column of creatinine solution expressed in millimeters. The most accurate results are obtained when the 500 c.c. of diluted urine contains from 7 mgs. to 15 mgs. of creatinine. Calculate the per cent. and the amount per day, assuming the volume of urine to be 1200 c.c.

513. Instead of the standard bichromate solution it is in many respects more satisfactory to use 1 mg. of creatinine. This standard solution is made by dissolving 1.6106 grams of the double salt, creatinine-zinc chloride (511), in a liter of decinormal hydrochloric acid. Eight c.c. then contains 1 mg. of creatinine. To make the colored standard, mix 1 c.c. of this creatinine solution with 20 c.c. of saturated picric acid solution and 1.5 c.c. of 10 per cent. sodium hydroxide, and after ten minutes dilute it to 100 c.c. This color is permanent at least twenty-four hours.

To determine the amount of creatinine in urine, measure 1 c.c. of urine with an accurate pipette into a 100 c.c. volumetric flask and 1 c.c. of the standard creatinine solution into another. Add to each 20 c.c. of saturated picric acid solution (measured with a cylinder) and 1.5 c.c. of 10 per cent. sodium hydroxide. After ten minutes fill to the mark with water and mix. Place the solutions in the two tubes of a colorimeter, set the standard at a definite height and compare the height of the other with it when the color is the same. The relative height represents the proportion of 1 mg. of creatinine present. If it is less than two-thirds or more than one and one-half times that of the standard it is better to repeat with a volume of the urine necessary to make them more nearly equal.

If no colorimeter is at hand place the colored solutions from the standard and the urinary creatinine in two Nessler tubes. Pour from the darker one until, when looking down into them on to a white background, they have the same shade. The relative heights of the liquids represent the reciprocals of the amounts of creatinine. That is, if the urinary sample is five-fourths the height

of the standard it will contain 80 per cent. as much creatinine as the standard.

514. The Determination of Creatine in Urine (Folin's method).—This depends upon converting the creatine into creatinine by boiling with an acid and determining the amount of the creatinine colorimetrically as in 512 or 513. It should follow that determination.

With an accurate pipette measure into a weighed Erlenmeyer flask of 200 c.c. capacity enough urine to give 0.7 to 1.5 mg. of creatinine, usually 1 c.c. Twenty c.c. of saturated picric acid, 130 c.c. of water and a few very small pebbles to promote even boiling are added and the mixture is gently boiled, preferably over a microburner, for an hour. At the end of this time the heat is increased and the solution boiled down to rather less than 20 c.c. The flask is transferred to the scales and water added to make 20 to 25 grams. The solution is cooled in running water, 1.5 c.c. of 10 per cent. sodium hydroxide is added and the color compared with that from 1 mg. of creatinine (513).

The result represents both the urinary creatinine and creatine. Subtraction of the amount of creatinine previously found gives the creatinine corresponding to the urinary creatine.

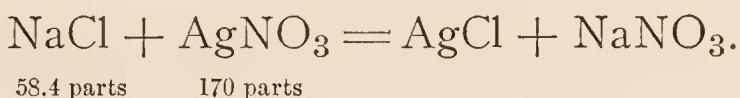
Write equation for change from creatine to creatinine. What is the function of the acid?

CHLORIDES.

In the urine the excreted chlorine, of which there is normally in a day 6 to 10 grams, is united principally with sodium. There is a small part with potassium as potassium chloride. The excretion of chlorides in health is increased with salt food and with large quantities of drink. Chlorides are necessary in the fluids of the body for the proper performance of their functions. When more chlorine is required by the body the chlorides are held back by the kidneys from the urine. When there is a less demand in the body the kidneys excrete the chlorides. Thus, in pneumonia and other diseases, where

there are serous exudations, the chlorides are withdrawn from the circulation to form the constituents of these fluids, as is shown by their decrease in the urine. When the pathological exudations are absorbed the amount of urinary chlorides increases. In fevers there is a decrease in the chlorides of the urine until the crisis, then an increase. In chronic diseases the amount of chlorine gives some indication of the digestive power, 6 to 10 grams per day being normal, and less than 5 grams daily showing subnormal metabolism, providing that an excessive amount has not been removed by other means, like serous exudations or diarrheic discharges. An excessive excretion of chlorine (15 to 20 grams daily) is found in diabetes insipidus. In dropsical conditions it is a favorable sign, showing the absorption of the fluid.

The quantity of chlorine can be determined by ascertaining how much silver nitrate is required to precipitate it.



To ascertain when the chlorine has all united with the silver a little yellow potassium chromate is added. The silver forms first a white silver chloride, and when the chlorine has been precipitated it forms the red silver chromate.

515. Acidify a portion of urine in a test tube with nitric acid and add a little silver nitrate. A white precipitate which turns dark in the sunlight indicates the presence of chlorides. Why is nitric acid used?

516. Determination of Quantity of Chlorine in Urine.—For clinical purposes the following method is sufficiently accurate: Measure with a pipette 10 c.c. of

urine and dilute with about 100 c.c. of water. Add a few drops of yellow potassium chromate solution; set the beaker in a rather dark corner where the chief illumination is from a yellow flame, such as a Bunsen burner with the air vents closed, then allow to flow into it from a burette a solution which contains 17.000 grams of silver nitrate in a liter. The change of color from white to red can be more plainly seen by yellow light (gaslight) than by daylight. As soon as the color of the precipitate changes from white to reddish, read off the volume of silver solution which has been used. Each cubic centimeter of this will precipitate 0.00354 gram of chlorine, equal to 0.00584 gram of sodium chloride. Calculate the percentage of chlorine by weight in the urine. There are present in the urine some other substances which are precipitated by silver nitrate like the chlorine. To make approximate correction for these, 1 c.c. may be subtracted from the volume used.

517. A more accurate result can be obtained if the organic matter is first destroyed. To 10 c.c. of urine in a thin porcelain or platinum dish add about 3 grams of sodium nitrate, free from chlorides. Evaporate to dryness and carefully heat to fusion. After it has deflagrated cool, dissolve in water, slightly acidifying with nitric acid, then make exactly neutral with sodium carbonate titrating with silver nitrate, and calculating the amount of chlorine as in the preceding experiment.

What is the action of the sodium nitrate?

PHOSPHATES.

The phosphoric acid of the urine is chiefly united with two classes of bases: the alkalies,—sodium and potassium,—and the alkaline earths,—calcium and magnesium. The compounds are called, respectively, “alkaline” and “earthy” phosphates. The alkaline phosphates are solu-

ble in water. The earthy phosphates are insoluble in water or alkalies, but are dissolved by acids. They consequently appear in the urine in the insoluble form whenever it becomes neutral or alkaline, either by fermentation or by the addition of reagents. They may also be precipitated by boiling. The amorphous white precipitate thus obtained is often mistaken for albumin. It can be distinguished by being easily soluble in acids, which is not the case with albumin. When ammonia is present, as in fermentation, the magnesium forms an insoluble salt with two bases, NH_4MgPO_4 . In urinary analysis it is referred to as triple phosphate. It is crystalline, sometimes in the form of snowflakes, but more commonly in prismatic crystals often spoken of as "coffin-lid crystals," from their supposed resemblance to the lid of a coffin. (Plate II, 8.)

The phosphoric acid of the urine is mainly that taken in the food, but a part comes from the oxidized phosphorus compounds of the tissues, such as lecithin and the nuclein compounds. The presence of a sediment of the earthy phosphates shows simply that the urine is alkaline, and is no indication that an excessive amount is being excreted. Animal foods are richer in phosphoric acid compounds than vegetable; hence with these we find more in the urine.

Experience has shown that there is a diminution of the excreted phosphoric acid in many pathological conditions. This is true in most acute infectious diseases, in nephritis, gout, and rheumatism. In diabetes mellitus and in some diseases of the bones there is an increase. Still, with the exception of the bones, the tissues of the body contain but comparatively small amounts of phosphorus compounds, and with our present knowledge it is difficult

to draw definite conclusions regarding the decomposition of such tissues from the variations in the eliminated phosphoric acid.

518. Make a specimen of urine alkaline with sodium hydroxide. The earthy phosphates are precipitated in an amorphous form. Examine under the microscope. See that they are dissolved again by acidifying with even a weak acid, like acetic. Sketch in note-book.

519. Filter out the earthy phosphates and test the filtrate for the phosphoric acid of the alkaline phosphates by adding magnesia mixture. (This is magnesium sulphate made alkaline with ammonia and enough ammonium chloride to dissolve the precipitate first formed.) With phosphoric acid or its salts it gives a white crystalline precipitate. Sketch in note-book.

520. Form triple phosphate by making urine very faintly alkaline with ammonia and allowing it to stand until the precipitate settles. Examine under the microscope for the "coffin-lid" crystals. They can be more abundantly formed for microscopic examination by adding to the urine a little of a solution of magnesium sulphate before making it alkaline with ammonia. If too much ammonia is used the crystals will be of the "snow-flake" type. Sketch typical forms in note-book.

521. **Determination of Amount of Phosphoric Acid.**— Prepare the following solutions:—

1. Uranium acetate: Dissolve about 34 grams of crystallized uranium acetate in water and dilute to one liter. This solution will be a little too concentrated. Its exact concentration must be found by the method to be described later.

2. A solution of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (crystallized disodium phosphate), one liter of which shall contain 10.085 grams of the pure crystallized salt. This salt gives up its water of crystallization when exposed to the air, and cannot then be used. The crystals must be perfectly bright. Fifty c.c. of the solution contain 0.1 gram of P_2O_5 (phosphoric anhydride).

3. Solution of sodium acetate, of which one liter contains 100 c.c. of 30 per cent. acetic acid and 100 grams of sodium acetate.

4. Solution of cochineal made by digesting for some time 1 gram of powdered cochineal in a mixture of 20 volumes of alcohol with 60 volumes of water. Filter or decant the liquid.

Operation.—First ascertain the concentration of the uranium solution. To accomplish this, measure with a pipette 50 c.c. of the sodium phosphate solution into a beaker; add 5 c.c. of the sodium acetate solution and a few drops of cochineal. Heat to boiling, and then from a burette run in the uranium solution, drop by drop, until a greenish color is produced. The phosphoric acid has then been precipitated. Since 1 c.c. of the uranium solution ought to precipitate 0.005 gram of P_2O_5 , exactly 20 c.c. should have been used for the 50 c.c. of sodium phosphate. If this is not the quantity which has been used, first ascertain accurately how much is needed and then dilute the uranium solution so that 1 c.c. precipitates 0.005 gram of P_2O_5 . If, for instance, 17.5 c.c. have been used instead of 20 c.c. there must be added 2.5 c.c. of water for every 17.5 c.c. of the uranium solution.

The amount of P_2O_5 in urine can now be determined in the same manner, using urine instead of the sodium phosphate solution. Calculate the percentage of P_2O_5

present, knowing that there is 0.005 gram for each cubic centimeter of uranium solution which has been used.

522. After acquiring the technique determine P₂O₅ in unknown samples of urine furnished by instructor. Report grams per day assuming 1000 c.c. of urine passed.

SULPHATES.

The sulphates of the urine are of two classes: (1) those of which the base is a metal, like K₂SO₄ and Na₂SO₄, and (2) those in which a part or the whole of the base has been replaced by an organic radical, like KC₆H₅SO₄. Those of the first class are called the inorganic, and the second organic, conjugate, or ethereal, sulphates. The latter differ from the inorganic in not forming an insoluble precipitate upon the addition of a barium salt as the inorganic do. The two classes can be separated by this means. After the removal of the inorganic sulphuric acid by barium chloride the organic sulphates can be decomposed by means of boiling hydrochloric acid:—

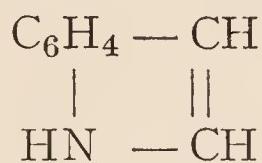


The sulphate will then give the white precipitate of barium sulphate if barium chloride be added.

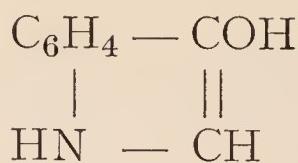
The total amount of combined sulphuric acid excreted by an adult in twenty-four hours is 2 to 3 grams. It is derived partly from that already formed in the food, which passes without change into the urine, but, for the most part, from the oxidation of sulphur compounds, like albumin, in the body. Variations in the total sulphuric acid in general indicate the rate of oxidation of sulphur compounds. It is increased by taking such compounds, *e.g.*, by a meat diet. It is decreased by a vegetable diet.

The organic sulphates normally make up about one-tenth of the total sulphates. The organic bases of these are such compounds as phenol (C_6H_5OH), cresol ($C_6H_4CH_3OH$), indoxyl (C_8H_6NOH), etc. These bases are formed by the putrefaction of albuminous substances; consequently, when such putrefaction is in progress in the body the organic sulphates increase in the urine. They may be formed in the intestine or absorbed from some other source. In the former case they are increased whenever there is a serious stoppage of the food, as in ileus or in peritonitis with atony of the intestine. In ordinary constipation there is no marked increase. In diseases which are accompanied by an internal suppuration there is an increased amount of organic sulphates in the urine, and this fact may be used to judge whether the pus-forming stage has been reached. This is the case in fetid bronchitis, carcinoma of the stomach or intestine, diphtheria, pyemia, etc. If the formation is from putrefaction in the intestine it will be diminished by taking antiseptic remedies, like calomel, or those which, by their purgative action, remove the contents of the intestine before this putrefaction has occurred.

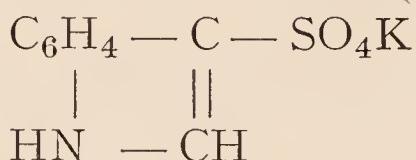
The compound of indol which is found in the urine goes by the name of indican. The indol,



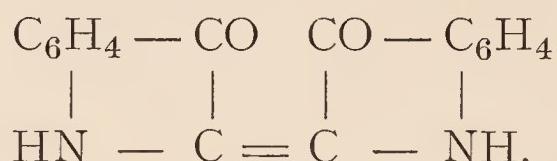
formed by the putrefaction of albuminous substances, is oxidized after it has been absorbed from the intestine or elsewhere in the body and becomes indoxyl:—



This unites with potassium and sulphuric acid to form indican:—



Indican may be easily oxidized by chlorine or other oxidizing agents, and then forms indigo blue:—



Putrefaction of nitrogenous compounds in the small intestine seems to be more productive of indican than when it goes on in the large intestine. Sometimes the indican is decomposed in the urine, the indigo being set free in the form of blue or red microscopic crystals. It is usually dissolved as a sulphate, however, until the indigo is formed by an oxidizing agent. It is normally present in large quantities in the urine of the horse, where, because of the long intestine, the residue from the food requires a considerable time to pass from the body.

523. Preparation of Potassium Phenyl-Sulphate ($\text{KC}_6\text{H}_5\text{SO}_4$).—First prepare, if it is not at hand, potassium pyrosulphate by mixing 25 grams of finely-powdered potassium sulphate with 15 grams of concentrated sulphuric acid, then heating (best in platinum dish, although a porcelain one can be used). The heating should be done under a hood, to avoid the acid fumes. The heat should be gently applied at first, stirring until all the crystals have dissolved. When it ceases to bubble increase the heat to low redness. Allow it to cool, but before it solidifies it is

best to carefully pour it upon a piece of clean sheet iron. Powder finely the potassium pyrosulphate ($K_2S_2O_7$) thus obtained.

In a thin glass flask holding about a liter dissolve 15 grams of potassium hydroxide in 20 or 25 c.c. of water, then add 25 grams of crystallized phenol (carbolic acid, C_6H_5OH). When it has dissolved let it cool to 60° or 70° C., and, while stirring well, add gradually in small quantities 30 grams of potassium pyrosulphate powdered as finely as possible. Keep it at a temperature of from 60° to 70° for from eight to ten hours, shaking often. Then add about 125 c.c. of boiling 95 per cent. alcohol, and filter while it is hot. This filtration is best performed in a hot water funnel—that is, one which is surrounded with a hot water jacket. Otherwise the salt will crystallize out before the liquid has passed through the filter. As soon as the filtrate cools, the potassium phenyl-sulphate crystallizes in pearly plates. It should be filtered out and recrystallized from a small quantity of boiling alcohol.

524. Test a solution of this organic sulphate with barium chloride. There is no precipitate. Compare the result with that obtained from an inorganic sulphate, like magnesium sulphate, with barium chloride.

525. Acidify a solution of an organic sulphate with hydrochloric acid, boil, and add barium chloride. The acid has decomposed the sulphate, so that a precipitate of barium sulphate is now obtained.

526. Show that a mixture of the two classes of sulphates, as in urine, can be detected in this way. First acidify by acetic acid, then, after adding barium chloride, let the test tube stand at least half an hour in a beaker of boiling water. The inorganic sulphates are thus precipitated as barium sulphate, but not the organic. Filter, and test the filtrate with a drop of barium chloride. If enough was added at first there will be no precipitate. If there is, more barium chloride must be used,

and the heating repeated. When the filtrate remains clear, acidify with hydrochloric acid and boil. The precipitate is from the decomposed organic sulphates united with the barium chloride previously added.

527. Explain different action of two classes of sulphates on basis of ionic theory.

528. Insert into a rabbit's stomach a wide, flexible catheter or rubber tubing, passing it through a short piece of glass tubing held between the animal's teeth. Introduce by this tube a gram of ortho-nitro-phenyl-propionic acid and collect the urine for twenty-four hours. It will contain a large quantity of indican. Read the literature on the relationship of the above acid to indigo, and explain the formation of indican.

529. Test urine for indican by acidifying strongly with commercial concentrated HCl. Then add a minute fragment of calcium hypochlorite ("chlorinated lime") and a few drops of chloroform and shake gently. Let the chloroform settle to the bottom. If indican is present in the urine it will be thus oxidized to indigo blue, and this colors the chloroform. A second piece of the hypochlorite may be added and the shaking repeated. An excess will destroy the blue color. Instead of calcium hypochlorite, a few drops of chlorine water, or hydrogen dioxide can be used. This is Jaffé's test.

530. Obermayer's reagent for indican contains 2 to 4 grams of ferric chloride in a liter of concentrated hydrochloric acid. An excess does not destroy the indigo. Mix equal volumes of the reagent and urine and shake. Indican is oxidized to indigo blue. This can be taken up by a drop of chloroform, as in the preceding test.

After the internal use of iodine compounds the iodine is excreted largely through the urine. The reagents of either Jaffé's

or Obermayer's tests will set it free and it will dissolve in the chloroform with a color which simulates or conceals the indican reaction. A few drops of sodium thiosulphate solution will, however, destroy the iodine color, but not that of indican.

531. Add to 10 c.c. of normal urine a few drops of a solution of potassium iodide and make Jaffé's or Obermayer's test, following this with sodium thiosulphate.

532. Quantitative Determination of Total Sulphates of Urine. Benzidine Method (Rosenheim and Drummond¹).—Inorganic sulphates are precipitated by a benzidine solution as benzidine sulphate which is quite insoluble. After this has been filtered off and washed its amount can be determined by titrating with a standard alkali, as the benzidine is a weak base and the compound is easily dissociated, the sulphuric acid then being neutralizable by the standard alkali.

In a mortar rub 4 grams of benzidine to a paste with about 10 c.c. of water and rinse it into a 2 liter volumetric flask, diluting to about 500 c.c. Acidify with 5 c.c. of concentrated hydrochloric acid, mix and dilute to the mark. One hundred and fifty c.c. of this solution is enough to precipitate 0.1 gram of the sulphate ion.

With a pipette measure 25 c.c. of urine into a 250 Erlenmeyer flask and drop in hydrochloric acid until the reaction is distinctly acid to Congo red paper (usually 1 to 2 c.c.), but avoiding an excess. Add 100 c.c. of the benzidine solution and let it stand for ten minutes. Filter with a Buchner funnel and wash with 10 to 20 c.c. of an aqueous saturated benzidine solution without allowing the precipitate to be sucked dry, in order to prevent its caking. Place the paper and precipitate in the same Erlenmeyer flask with about 50 c.c. of water, heat and

¹ Biochemical Journal, 1914, viii, 143.

titrate with decinormal sodium hydroxide using phenolphthalein for an indicator.

One c.c. of decinormal sodium hydroxide corresponds to 4.9 mgs. of sulphuric acid or 4.8 mgs. of SO_4 .

If it is desired to determine the amount of organic sulphates also measure 25 c.c. of urine into the flask as before, add 20 c.c. of 10 per cent. hydrochloric acid and boil gently for fifteen to twenty minutes. Then neutralize carefully and drop in hydrochloric acid until the reaction is acid to Congo red. The organic sulphates are hydrolyzed and the precipitation and titration is then carried out as above. This gives the total sulphate ion concentration. Subtracting from this total the amount of the inorganic sulphate ion found in the first determination gives the concentration of the organic sulphate ion.

Calculate the weight of each in 1000 c.c. of urine.

PROTEINS OF THE URINE.

The principal simple protein occurring in the urine is serum-albumin. Besides this there may be found there serum-globulin, proteose, fibrin, and possibly peptones. The nucleoproteins also are not uncommon, being often mistaken for mucin.

Albuminuria.

Serum-albumin may find its way into the urine either from the kidneys (renal albuminuria) or from serous liquids,—like blood, pus, or lymph,—mixing with it at some point in the urinary tract below the kidneys. When it is due to degenerative changes in the kidney it is usually accompanied by epithelium from the tubules, often in the form of cylinders or casts. Changes in the

composition of the blood or in the blood pressure may allow albumin to pass through the kidney. This is seen in anemic conditions, after some poisons, and in some infectious diseases, the kidneys in any of these cases not being necessarily in a pathological state. Severe muscular labor may cause the temporary appearance of albumin. The quantity present varies greatly under different conditions, and is not necessarily a measure of the severity of the disease. Still comparative tests in the same case will indicate something of its progress.

The amount of albumin in the urine can be determined accurately by precipitating, drying, and weighing. For a practical test, sufficient to show the variation in amount, Esbach's method can be used. This depends upon precipitating the albumin with a solution containing 1 per cent. picric acid and 2 per cent. citric acid. The operation is performed in a graduated test tube, called an albuminometer, the height of the precipitate indicating its amount. Variations in temperature greatly affect the height of the precipitate; consequently in comparative determinations the conditions of temperature must be always the same. The results are most accurate when not more than four grams of albumin are contained in a liter.

Tests for Albumin in the Urine.

If the urine is not clear it must be filtered before testing.

533. The Heat, or Boiling, Test.—Heat the urine to boiling in a test tube, then acidify with a few drops of concentrated nitric acid. If albumin is present, a white precipitate remains. The earthy phosphates precipitate on boiling, but are soluble in acids.

534. **The Ring Test** (Heller's test).—Pour half an inch of concentrated nitric acid into a narrow test tube. From a pipette, the end of which is held just above the surface of the acid, drop the urine slowly or hold the tube in a slanting position and slowly pour upon the acid an equal volume of urine. If albumin is present a white cloud forms at the point of contact of the two liquids. If the amount is exceedingly small, it may not appear for half an hour. (If biliary pigments are present the ring may be colored.)

535. Acidify 2 or 3 c.c. of potassium ferrocyanide solution with about 1 c.c. of acetic acid, and fill the test tube half full of urine. Albumin gives a white, cloudy precipitate. An excess of ferrocyanide interferes with the accuracy of the test.

536. Add to the urine in a test tube about one-sixth of its volume of a saturated solution of sodium chloride, acidify with acetic acid, and boil the upper part of the liquid, holding the tube by the bottom. Albumin gives a white precipitate, which shows plainly above the clear liquid in the lower part of the tube.

To each of these tests are some objections which must be recognized in interpreting the results. By the action of heat and nitric acid some of the albumin is decomposed; hence the first test is not as sensitive as some others. This decomposition is greatly increased if the urine is boiled after adding the acid. Besides albumin, there may be precipitated by this test uric acid from the urates in very concentrated normal urine, and also resinous matters after the administration of turpentine or the balsams. The resinous compounds are soluble in alcohol, which does not dissolve albumin. The uric acid compounds are colored instead of being white, like albumin, and can be filtered out and tested.

The ring test with nitric acid is very sensitive. It precipitates other substances than albumin,—such as the urates, mucin, and resinous substances. The urates do not form a ring at the plane of contact of the two liquids, but above it; and if the urine is previously diluted with two or three times its volume of water they do not appear. The resinous matters dissolve in alcohol. The mucin precipitate forms a cloud in the upper part of the liquid where the acid is dilute. It dissolves in concentrated nitric acid.

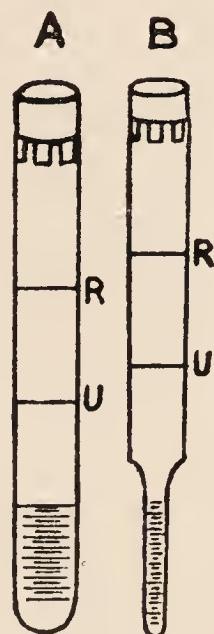


Fig. 13.—Esbach's albuminometer. *A*, usual form. *B*, form for minute quantities.

Potassium ferrocyanide and acetic acid will detect very small quantities of albumin. Proteose is also precipitated if present. If the acid alone produces a cloudiness it is mucin or resinous compounds. These must be removed by filtration before adding the ferrocyanide.

In the sodium chloride and acetic acid test the precipitate formed on boiling is acid albumin, which is insoluble in the salt solution. Resinous matters may be precipitated, but not mucin.

537. Quantitative Determination of Albumin in Urine (Esbach's method).—The urine must not have a specific gravity above 1.008, otherwise it must be

diluted with a definite volume of water. If it is not distinctly acid in reaction it must be made so by acetic acid. Fill the albuminometer (Fig. 13) with urine to the mark *U*. Add the reagent to the mark *R*. Close with a cork and mix gently, avoiding hard shaking, which introduces air bubbles into the precipitate and thereby prevents its settling. Let it stand at the temperature of the room (60° to 70° F.) for twenty-four hours. The height of the precipitate indicates the number of grams of albumin per liter, or parts in a thousand.

538. Quantitative Determination of Urinary Albumin.
Gravimetric (Folin and Denis).—Pipette 10 c.c. of the urine into a dry, weighed, conical centrifuge tube, add 1 c.c. of 5 per cent. acetic acid and let the tube stand 15 minutes in a beaker of boiling water. At the end of this time centrifuge the tube a few minutes; then pour off the supernatant liquid, stir up the precipitate with about 10 c.c. of boiling 0.5 per cent. acetic acid and centrifuge again. Again pour off the supernatant liquid and stir with 50 per cent. alcohol. Centrifuge the third time and after pouring off the liquid dry the tube with the precipitated albumin for two hours in a drying oven at 100° to 110° . Cool in a desiccator and weigh. (Any globulin present in the urine will be coagulated and weighed with the albumin in this process).

Would any of the following interfere with the accuracy of the determination,—blood, glucose, mucin, peptones?

539. Determination of Urinary Albumin (Kjeldahl).—Acidify 100 c.c. of urine with a few drops of acetic acid, avoiding an excess, then heat until the albumin coagulates. Filter through a nitrogen-free filter, wash with hot water and determine the nitrogen in the precipitate, without separating it from the paper, by Kjeldahl's method (490). Multiply the N by 6.25 to get the weight of coagulable protein.

540. Determination of Amount of Precipitable Urinary Protein by Nephelometry (Folin and Denis's method).¹—Nephel-

¹ Journal of Biological Chemistry, 1914, xviii, 273.

ometry is a method of determining small amounts of a precipitated substance by the degree of turbidity. It can be used only where the precipitate does not settle or aggregate rapidly.

Colorimeters can be modified for this purpose. One convenient form is Bloor's modification of the Duboscq instrument.² The changes made are the removal of the glass prism plungers,

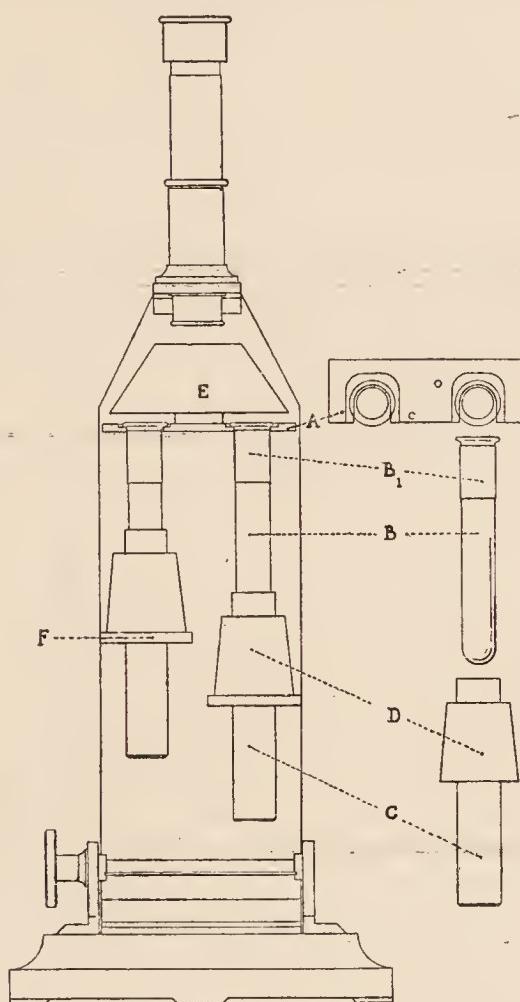


Fig. 14.—The Duboscq colorimeter converted into a nephelometer, showing the necessary extra parts.

substitution of opaque jackets for the movable cups and use of light from the front instead of from below.

This nephelometer is shown in Fig. 14 (compare with the Duboscq colorimeter, Fig. 9). The brass plate carrying the prisms is replaced by plate *A* with two slots in which are supported, by their flanges, the nephelometer tubes *B*. The colori-

² Journal of Biological Chemistry, 1915, xxii, 145.

meter cups are replaced by the closed-bottom jackets *C* which are made of metal or glass painted light-tight, and which are supported on the cup supports *F* by the collars *D*. The nephelometer tubes are small test tubes 100 x 15 mm., preferably made from the same sample of colorless glass tubing so that they shall have exactly the same bore. To avoid possible differences they should be always used, like the jackets, on the same side.

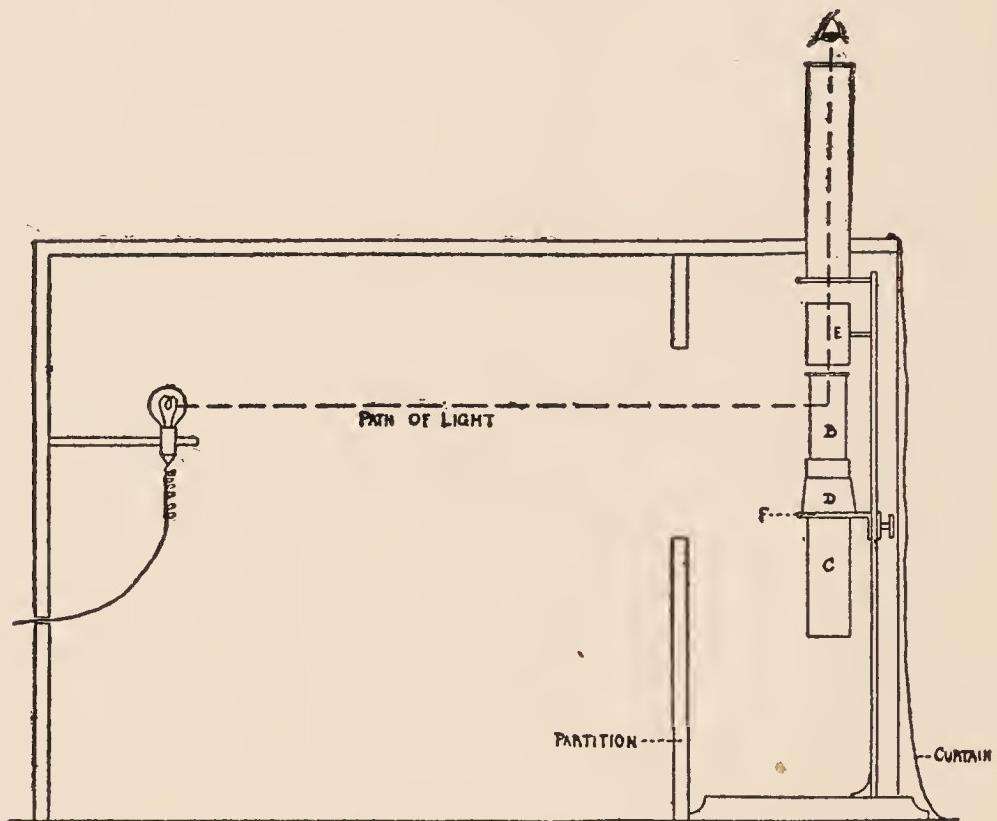


Fig. 15.—Nephelometer in box showing its position with reference to the source of light.

Insert the tubes and move up the jackets until the indicator on the scale is at zero, then mark the tube at the point reached by the top of the jacket *B* and make it opaque above this point by black paper or paint. For illumination use a 50 watt tungsten supported 30 cm. in front of the nephelometer and at the level of the tubes. The whole is surrounded by a box with a partial partition (Fig. 15) which shuts off light from the bottom of the instrument. It can be made without a bottom and provided with a curtain behind and it is painted black inside.

In using the nephelometer put the unknown in one tube and a precipitate made under the same conditions from a solution of

known concentration in the other. Turn the screw until the brightness of the two is the same and calculate the relative amounts in the two tubes from the difference in the length illuminated. If the concentrations are within 10 to 15 per cent. of each other they can be regarded as inversely proportional to the scale readings. If they vary greatly, a correction is necessary. Kober¹ has proposed an equation for such correction but it is simpler to make the two solutions nearly the same before taking the reading.

To prepare the standard protein solution take fresh blood serum, free from hemoglobin, and dilute 25 to 35 c.c. to about 1500 c.c. with a 15 per cent. solution of chemically pure sodium chloride. Filter the mixed solution and by Kjeldahl's method (490) determine the amount of protein (protein = N × 6.25). Then dilute with 15 per cent. salt solution so that 1 c.c. contains 2 mg. of protein. If it is saturated with chloroform by shaking with a few drops it will keep for months.

To precipitate the protein take, in each of two volumetric flasks about 75 c.c. of water and 5 c.c. of a 25 per cent. solution of sulphosalicylic acid. To one flask add by an Ostwald pipette 5 c.c. of the standard protein, to the other the albuminous urine (filtered if it is not clear) in portions of 1 c.c. each until the turbidity is nearly that of the standard. Fill the flasks to the mark with water and mix by carefully inverting a few times. Pour them into the two nephelometer tubes, setting the standard at 20. Compare their brightness by the method described above, taking the mean of several readings. The unknown reading must be between 10 and 30, better between 15 and 25. Calculate the amount of protein in 1000 c.c. of urine.

Globulin, Proteose, and Peptones.

Globulin is found in the urine only with albumin. It passes into the urine in much the same manner, and has no different diagnostic value.

Proteose may be formed in urine from albumin by bacterial action. It may easily escape discovery,

¹ Journal of Biological Chemistry, 1913, xiii, 485.

since it is not coagulated by heat. It is often the precursor of albumin, and, as such, a knowledge of its presence is important. Before testing for its presence the other albuminous substances must be removed. After removing albumin and globulin by boiling the liquid slightly acidified by acetic acid, proteose can be detected by its giving a precipitate upon saturation with sodium chloride, which dissolves on heating and reappears on cooling.

541. To 10 c.c. of the clear urine (filtered if necessary) add an equal volume of a saturated solution of ammonium sulphate. The serum globulin will be precipitated, but not the albumin.

542. Saturate the clear urine with finely powdered magnesium sulphate without warming. Serum globulin is precipitated. This can be removed by filtration and confirmed by the usual tests for globulins.

The results of late research have shown that much of what has been regarded as peptone in urine is one of the proteoses which closely resembles it, and it is an open question whether peptones are ever found in this excretion. Nevertheless, we may temporarily retain the name peptonuria for the condition, with the understanding that, as our knowledge becomes greater, it may have to be abandoned. The peptones or proteoses are not normally found in the blood, being converted into another protein. When anything interferes with this conversion, or when they are otherwise introduced into the blood, they pass into the urine. Diseases of the intestine, like carcinoma or ulceration, give rise to *enterogenic* peptonuria. Peptone and proteose are formed by the decomposition of simple proteins by other means than by

digestion; as, for example, by putrefaction. Diseases which are characterized by a formation of pus are often accompanied by peptonuria. This is the so-called "pyogenic peptonuria." It is found when there is much formation of pus in a body cavity, as in croupous pneumonia and with deep-seated abscesses.

543. Proteoses and peptones can be tested for as follows:—First, test small portions of the urine for coagulable proteins by the boiling test (533) or Heller's test (534), and if they are present remove them by acidifying the urine with acetic acid, boiling and filtering. To the filtrate apply the biuret reaction, avoiding an excess of the copper solution. A positive result indicates proteoses (or possibly peptones).

543a. Heat 50 c.c. of urine to boiling; acidify if necessary with a few drops of acetic acid, filtering if it precipitates, and, while hot, add powdered ammonium sulphate to the filtrate as long as it dissolves and until there are some crystals in the bottom. Filter after cooling. This leaves the peptone in solution. To insure complete precipitation of the other proteins the saturation with ammonium sulphate may have to be repeated. When this has been done and no further precipitate results, test portions of the filtrate with (1) tannic acid with twice its volume of water; (2) potassio-mercuric iodide. Each should give a yellowish-white precipitate. The biuret test can be tried, but is not as sensitive as the others. With peptones, if no excess of copper sulphate is used, it should give a pink with no shade of blue. A large amount of sodium hydroxide must be used because of the ammonium salt present.

544. How does the presence of ammonium sulphate modify the biuret test? If it were present and protein absent what would be the result of the test? Equations?

Mucinuria.

Both normal and pathological urine often contain a substance which, although similar to true mucin, yet differs from it in many respects. On account of this resemblance it is often called urinary mucin. The latest investigations indicate that it is a nucleoprotein. In normal urine it appears after standing as a light, fleecy cloud in the middle of the liquid. Its origin is the mucous membrane,—principally that of the bladder, ureter, or vagina. In small amounts it has no special significance. In catarrhal inflammation of the bladder it is abundant. In cystitis and pyelitis it may give the urine a gelatinous appearance. Mucin is also increased in febrile conditions, as well as in nephritis.

Urinary mucin is precipitated from its solution by alcohol or dilute acetic acid without heating. It may be precipitated by very dilute mineral acids, but dissolves in excess. After precipitation by acids it is soluble in alkalies. Since nucleoproteins, like the mucin of the urine, are composed of a simple protein with a nucleic acid, they give most of the reactions of the albumins, such as those with potassium ferrocyanide, picric acid, the biuret test, etc. Care is necessary, therefore, to avoid confusing urinary mucin with small quantities of albumin. They can be differentiated by the fact that the mucin is precipitated in the cold by acetic acid even after the urine has been diluted with water, while albumin is not.

545. Dilute normal urine with its own volume of water, acidify a small beakerful with acetic acid and allow to stand to see if the mucin separates.

546. Show that the mucin dissolves by adding a few drops of an alkali, like sodium hydroxide, and that it is

reprecipitated from this solution by acidifying again with acetic acid. Is there any normal constituent of the urine which has the opposite solubility and which might therefore interfere with the test unless the mucin is first filtered out?

547. If urine containing much mucin can be obtained, apply the general tests for simple proteins. To what ones does it respond?

Fibrinuria.

Through hemorrhage or exudation of serous fluids into the urinary passages the urine sometimes becomes mixed with fibrinogen, and this may form clots or semigelatinous masses. It may cover the bottom of the vessel or occasionally cause the whole mass to gelatinize. The fibrin can be filtered from the liquid through muslin. It is very similar to the deposit of pus from fermenting urine. The pus, however, can be thinned with water. The fibrin is insoluble.

GLYCOSURIA.

Glucose is not normally found in large amounts in the urine, although traces are frequently—and perhaps always—present. More than a slight trace may be regarded as pathological if it continues for any length of time. A transitory form of glycosuria (alimentary glycosuria) is often caused by excessive quantities of sugar in the food. It may be produced by puncture of the fourth ventricle of the brain, by injuries of the pancreas, by a number of medicinal substances which act upon the vaso-motor nerves, such as phloridzin, etc.

The urine is generally, though not always, of a high specific gravity (1.030 to 1.050), having a light color and a whey like odor. The daily volume may be in-

creased to ten times the normal. When poured or shaken it retains the foam for a considerable time.

The tests commonly used to detect glycosuria are based upon the power of the sugar to reduce metallic oxygen compounds, its power to unite with phenyl-hydrazine in a characteristic compound, its property of fermentation with yeast, or, less frequently, its strong dextro-rotatory action. With the exception of the phenyl-hydrazine reaction all are likewise used to determine the quantity of dextrose present.

548. Test diabetic urine with

- A.* Trommer's test (40).
- B.* Fehling's test (42); Benedict's test (46); or Folin's test (48).
- C.* Bismuth subnitrate test (51) or Nyländer's test (52).
- D.* Phenyl-hydrazine test (54).
- E.* Other tests specified by instructor.

Notice that the other urinary constituents may modify the results obtained with a solution of pure glucose.

No one of the above tests is an absolute proof of the presence of glucose. Other constituents of urine have a slight reducing power, and may respond to the tests with alkaline solutions of copper or bismuth, where the action is that of reduction, for example, uric acid and its salts; creatinine, mucin, and others occurring in smaller amounts have this power of reduction and will reduce metallic compounds in alkaline solution. The same is true of many medicines which pass into the urine. Trommer's and Fehling's tests are very sensitive under ordinary conditions, but they may fail in some decomposing urines, the ammonia which is present keeping the cuprous oxide in solution. Long boiling will expel the ammonia, and the test may then succeed. Large amounts of uric acid, creatinine, or albumin may act in the same manner, keeping the red oxide from precipitating.

It must be borne in mind that the earthy phosphates will always be precipitated when the urine is made alkaline, and consequently, appear in many of the glucose tests. They are always colorless, as can be shown by washing them on a filter, whereas the oxide of copper or the bismuth reduced by the sugar are colored.

In the test with the subnitrate of bismuth the salt is not so easily reduced by other compounds than glucose. Consequently there is not so much danger of mistaking these for sugar. With a very large excess of the alkali this reduction may occur. This is said not to be the case with Nyländer's modification of the test (52). Albumin is, however, decomposed under such circumstances, giving a black precipitate. It must, therefore, be removed from the solution before the test is made. With this test very small quantities of sugar can be detected. Many medicinal substances pass into the urine and react with this test also.

The phenyl-hydrazine test is not affected by the reducing matters of the urine, but it gives a similar precipitate with milk-sugar. Pure phenyl-hydrazine must be used. If it is the hydrochloride, the crystals should be white, not brown.

The fermentation test is not very sensitive. It may be interfered with by the presence of some drugs which stop the action of the yeast. If the urine is not acid, it should be made faintly so with tartaric acid. It can be used to distinguish between glucose and lactose. Barfoed's test (49) may be employed for the same purpose.

Quantitative Determination of Urinary Glucose.

549. Determine the quantity of glucose in urine, using Fehling's solution (58). Dilute the urine with a measured volume of water if less than 5 c.c. of urine is required to reduce 10 c.c. of the reagent, and run in the diluted urine from a burette as with the pure sugar solution.

550. Use Folin and McEllroy's quantitative reagent to determine amount of glucose in urine (60).

551. Try Benedict's quantitative reagent (59) for glucose and compare the three titration methods as to desirability.

552. If it can be done test a sample of diabetic urine with the polariscope (61). Calculate the amount of sugar and compare the method with the others as to ease, accuracy and speed.

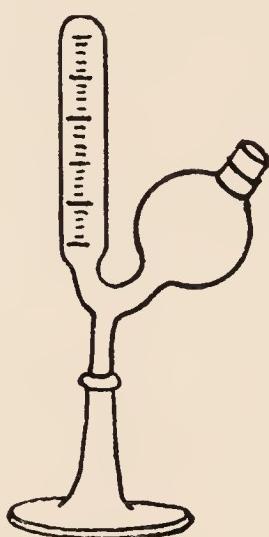


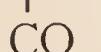
Fig. 16.—Einhorn's saccharimeter.

553. To 15 c.c. of neutral or acid urine in a test tube add a piece of compressed yeast as large as a pea, macerate with a glass rod and mix thoroughly. Place the liquid in a fermentation tube (saccharimeter Fig. 16). For very small amounts of sugar it is best to exclude the air with a drop of mercury. Allow the tube to stand in a warm room or incubator twelve to twenty-four hours. Read from the tube the percentage of glucose corresponding to the carbon dioxide evolved.

554. What do you regard as the advantages and disadvantages of the methods tried for determining the amount of urinary glucose?

THE ACETONE BODIES.

There are commonly included in this group acetone,



and beta-hydroxybutyric acid,

They are found during defective metabolism, as in advanced stages of diabetes, when there is a tendency toward an increase of acid products relatively to the alkalies of the blood (acidosis).

Acetone ($\text{CH}_3)_2\text{CO}$.

Normally acetone is present in the urine only in traces. Pathologically it occurs there in severe diabetes, in fevers, in inanition, and cachectic conditions, as well as in psychoses. In diabetes it often is a precursor of diacetic acid, which is significant of graver conditions. It appears to be formed by the decomposition of protein compounds, and it can be produced in the urine by the use of a diet of such substances. It is a colorless liquid of a fruity odor, which boils at 56.5° C . and which can consequently be readily distilled from the urine. The

examination of the urine should be made while it is fresh.

If a large quantity of acetone is present in urine the latter may be tested directly. Usually it is best to separate the acetone from the non-volatile urinary constituents which may interfere with the tests. This can be done by distillation after slightly acidifying with dilute sulphuric acid. A Liebig's condenser can be used or, if

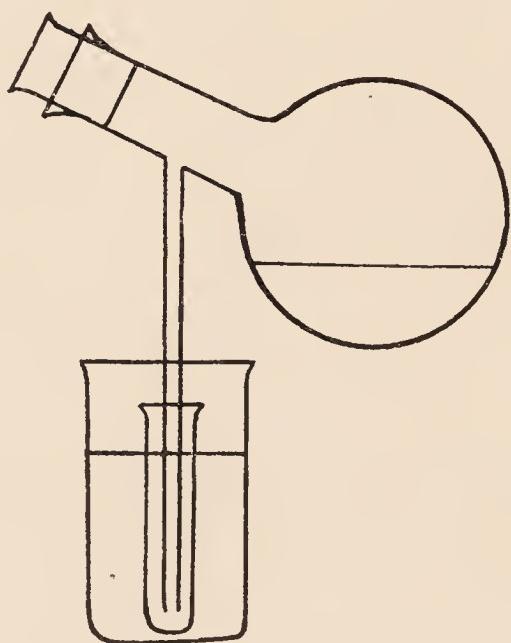


Fig. 17.—Arrangement for condensing acetone from urine.

but a limited amount of urine is available, the vapor from the distilling flask can be condensed directly in a test tube surrounded by cold water (Fig. 17). Because of its relatively low boiling point, distillation of one-tenth of the urine will drive out most of the acetone. If it is desirable to further concentrate it, this distillate can be redistilled and the first part of the second distillate used.

555. To a solution of acetone add a little sodium hydroxide, then a solution of iodine in potassium iodide

(Lugol's solution) and warm. Iodoform is produced as a yellowish powder having a characteristic odor (Lieben's test). After a time it may form six-sided plates, which can be seen with a microscope. Notice also the odor. Will any other compound give this reaction? (56.)

556. Is alcohol ever found in urine? Could it be found in diabetic urine on standing? Explain.

557. To 3 to 5 c.c. of the distillate from urine add a few drops of the iodine solution and make alkaline with a few drops of dilute ammonium hydroxide, instead of the sodium hydroxide. A black precipitate of nitrogen iodide forms which upon standing slowly changes to iodoform. Alcohol does not give this reaction. This is Gunning's modification of the iodoform test. Note the odor. Decant the liquid, dissolve the precipitate in a few drops of alcohol and allow this to evaporate on a microscope slide. Note the characteristic shape of the hexagonal, tabular yellow crystals of iodoform. Sketch these.

558. To the liquid containing acetone add a drop of a freshly prepared solution of sodium nitroprusside and make alkaline with sodium hydroxide. A ruby red color is produced. In a few minutes it changes to yellow. If, while red, it is acidified with acetic acid a carmine or purplish red color appears if much acetone is present. On long standing (forty-eight hours) this changes to blue (Legal's test). In cases of doubt the urine may be distilled and the acetone sought in the distillate.

559. How does this reaction compare with that of nitroprusside on creatinine (509)? How can they be differentiated?

560. Make about 10 c.c. of urine (or 1 to 2 c.c. of the distillate) strongly alkaline with sodium hydroxide,

add 10 to 12 drops of a 10 per cent. alcoholic solution of salicylaldehyde and warm to about 70°. In the presence of acetone the mixture turns yellow, then light red, reddish purple, and dark red.

561. Add to 3 c.c. of clear urine (filtered if necessary) 1 c.c. of a 3 per cent. peptone solution; then Lugol's solution (iodine in potassium iodide) until the color is a deep reddish brown, and, lastly, about 2 c.c. of ammonium hydroxide, and mix. A brownish black color should appear and last about ten minutes. If it disappears within a minute after adding the ammonia, too little iodine solution was used. After an hour and a half acidify with hydrochloric acid; if the liquid is clear, no acetone is present. If after acidifying a brown color remains from an excess of iodine, this can be removed by a few drops of very dilute sodium thiosulphate solution. If acetone is present there should remain, when seen with the microscope, an abundance of needle shaped crystals, singly or in clusters. With traces of acetone the liquid may need to stand an hour for crystallization to occur (Bardach's test).

The test is very sensitive and can be made in the urine directly without previous distillation. Aceto-acetic acid gives the same result. If sugar is present more iodine solution will be necessary.

562. Prepare mercuric oxide by precipitating a little mercuric chloride with sodium hydroxide. Wash by decantation, filter and wash. Add this to some of the acetone solution, shake, and filter. The presence of acetone is shown by its dissolving the oxide. This can be proved by pouring a layer of ammonium sulphide solution on top of the filtrate in a narrow test tube, when mercuric sulphide will be precipitated as a black ring between the two liquids.

563. **The Quantitative Determination of Urinary Acetone: Colorimetric (Csonka's method)**¹.—Three solutions are necessary:

A standard solution of acetone containing 0.1 mg. in 2 c.c.

¹ Journal of Biological Chemistry, 1916, xxvii, 209.

A solution of 10 grams of salicylaldehyde in enough alcohol to make 100 c.c.

A solution of 100 grams of potassium hydroxide in 60 c.c. of water. Cool before using.

Measure accurately with a pipette 25 c.c. to 100 c.c. of urine into a 750 c.c. flask, add 5 c.c. of concentrated sulphuric acid and dilute to about 300 c.c. Connect with a Liebig's condenser with the outlet dipping into about 25 c.c. of water in the receiver and distill twenty minutes. Transfer the distillate to a 200 c.c. volumetric flask and dilute to the mark.

Take 2 c.c. of the distillate in each of two large test tubes for duplicate determinations and at the same time treat similarly 2 c.c. of the standard acetone.

To each tube add 2 c.c. of the KOH and 1 c.c. of the salicylaldehyde. Exact measurements are necessary. Place the tubes together in a water bath at 45° to 50° for exactly twenty minutes to develop the color, during which time they should be mixed several times. Then add 10 c.c. of distilled water to each, cool, place in 25 c.c. volumetric flasks, fill to the mark and compare the colors with that of the standard within thirty to forty-five minutes of addition of aldehyde. If a Duboscq colorimeter is used set the standard at 15 mm. In absence of a colorimeter, Nessler tubes can be employed.

Diaceturia.

Diacetic or aceto-acetic acid ($\text{CH}_3\text{COCH}_2\text{CO}_2\text{H}$) never appears normally in the urine in more than traces, but is found under the same pathological conditions as acetone. In the fevers of childhood it is not so dangerous, but with adults it signals the approach of coma, of which it is, perhaps, one of the causes, through lowering the alkalinity of the blood. Diacetic acid is a colorless, strongly acid liquid, soluble in water and ether. On heating it decomposes below 100° to acetone and carbon dioxide:—



With ferric chloride it gives a violet red solution, which disappears on standing twenty-four hours and more quickly upon boiling. This reaction can be used to detect diacetic acid in the urine. A number of other substances —like salicylic and carbolic acids, antipyrin, and the acetates—give a somewhat similar reddish color. These are stable at ordinary temperatures, and only that from the acetates is decomposed by boiling. The tests should be made upon urine which has been comparatively freshly passed.

564. Test fresh urine for diacetic acid by adding, drop by drop, a solution of ferric chloride as long as a precipitate forms. This is ferric phosphate, formed from the phosphates of the urine. Filter, and to the filtrate add a few drops more of ferric chloride. The diacetic acid gives a violet red color. Allow it to stand several hours, and notice that it fades and disappears.

565. If this violet red color was obtained, boil another portion of urine for five to ten minutes, and after cooling repeat the test. If the red color was caused by diacetic acid less or none will be obtained in this second test, since the acid will have been decomposed by boiling.

566. **Aminoacetophenon Test.**—Two solutions are used: A 1 per cent. solution of para-aminoacetophenon with enough dilute hydrochloric acid dropped in to make it colorless, but carefully avoiding an excess; also a 1 per cent. solution of sodium nitrite.

Before using mix two volumes of the acetophenon solution with one of the nitrite, add an equal volume of the urine and a few drops of ammonia. A brick red color appears. One c.c. of this with 10 c.c. of concentrated commercial hydrochloric acid gives a purple violet color if acetoacetic acid is present. Instead of commercial hydrochloric acid the pure acid with a few drops of ferric chloride can be used. If the urine to be tested is highly

colored, it can first be mixed with a pinch of animal charcoal and filtered. The color can be separated from the liquid by a drop of chloroform, as in the indican test (529). This test does not respond to the benzene compounds commonly used as antipyretics, nor to acetone, carbolic acid, or salicylic acid.

567. The Determination of Acetone, Acetoacetic Acid and Betahydroxybutyric Acid (Van Slyke)¹.—The underlying principles are (*a*) acetone is precipitated by mercuric sulphate, (*b*) acetoacetic acid is converted into acetone by heating, (*c*) betahydroxybutyric acid is converted into acetone by oxidizing agents. Write equations for these changes.

After the removal of glucose and other interfering substances boiling with mercuric sulphate gives an insoluble compound of the free acetone and that derived from the acetoacetic acid. Boiling with mercuric sulphate plus the oxidizing agent gives an insoluble compound containing acetone from the hydroxybutyric acid as well as from the two other sources.

Solutions Required.—Twenty per cent. copper sulphate, made by dissolving 200 grams of crystallized copper sulphate in water and diluting to 1 liter.

Ten per cent. mercuric sulphate, made by dissolving 73 grams of pure red mercuric oxide in 1 liter of 4N sulphuric acid.

Fifty volume per cent. sulphuric acid; that is, 500 c.c. of concentrated acid, sp. gr. 1.835 diluted to 1 liter.

Ten per cent. calcium hydroxide suspension; that is, 100 grams of fine, light "reagent" hydroxide (Merck's) shaken up in 1 liter of water.

Five per cent. potassium dichromate;—50 grams of the dichromate in water, diluted to a liter.

Removal of Glucose and Interfering Substances.—Place 25 c.c. of urine in a 250 c.c. volumetric flask and mix with 100 c.c. of water and 50 c.c. of the copper sulphate solution; then add 50 c.c. of the calcium hydroxide suspension, and shake. If not alkaline to litmus add more of the hydroxide. Dilute to the mark and let it stand at least one-half hour to precipitate the glucose. Filter through a dry folded filter and use the filtrate for test-

¹ Journal of Biological Chemistry, 1917, xxxii, 455.

ing. (If the urine contains more than 8 per cent. of glucose it should be diluted to this amount before precipitating.)

Determination of Total Acetone Bodies.—Place in a 500 c.c. Erlenmeyer flask 25 c.c. of the urinary filtrate; add 100 c.c. of water, 10 c.c. of the 50 per cent. sulphuric acid and 35 c.c. of the mercuric sulphate solution. Connect with a reflux condenser having an 8 to 10 mm. diameter straight condensing tube and heat to boiling. After the boiling has begun pour through the condensing tube 5 c.c. of the 5 per cent. dichromate solution. Boil gently one hour and a half. Filter the precipitate in a Gooch or alundum crucible, wash with 200 c.c. of cold water, dry for an hour at 115°, cool in the room and weigh. Twenty mg. of the precipitate represents 1 mg. of acetone.

Determination of Betahydroxybutyric Acid.—Place 25 c.c. of the urinary filtrate after removal of glucose in a flask with 100 c.c. of water and 2 c.c. of the 50 per cent. sulphuric acid and boil with the flask open for ten minutes when the acetoacetic acid will have been decomposed and all the acetone distilled out. Measure the liquid in a cylinder, return it to the flask and rinse the cylinder with enough water to make the total 127 c.c. Then add 8 c.c. of the 50 per cent. sulphuric acid and 35 c.c. of the mercuric sulphate and complete the determination by oxidizing with dichromate as before; 8.45 mg. of the precipitate represents 1 mg. of betahydroxybutyric acid.

Determination of Acetone and Acetoacetic Acid.—Carry out the determination as for the total acetone bodies except that no dichromate is added and the boiling is continued not less than thirty, nor more than forty-five minutes. Under these conditions the betahydroxybutyric acid is not affected.

LACTOSURIA.

Milk sugar may be found in the urine of women toward the end of pregnancy and a short time after child-birth. Its presence indicates the absorption of the sugar from the fluid in the mammary gland. It may appear with the interruption of nursing or from stagnation of the milk in the gland. When the gland is well developed

and lactose is found in the urine during the period of nursing it shows merely that the secretion of milk is abundant. The chemical reactions of lactose are very similar to those of glucose. The principal differences are that lactose ferments with yeast with difficulty or not at all, and that its power of reduction is less than that of glucose. Still, the distinction between the two as they occur in urine is a matter of some difficulty.

568. Try the fermentation test with compressed yeast, as in 56, upon urine containing glucose and that containing lactose, and notice that the former ferments, with the evolution of carbon dioxide, and the latter does not.

569. Try Barfoed's test (49) upon the two kinds of urine, and notice that it responds to glucose, but not to lactose.

570. Which of the common sugar tests would give the same results with glucose and lactose? How would you determine its amount?

CHOLURIA.

In examining the urine for bile two classes of compounds are sought for: the biliary acids and the biliary pigments. The biliary acids do not normally occur in urine, except in small amounts. The pigments are more commonly found. In the freshly passed urine usually only bilirubin is present, but by oxidation it may be changed to biliverdin, etc.: Urine which contains bile is generally of a yellowish to greenish brown color, and the sediment, if it contains epithelial cells, is often colored brown. Upon shaking the urine the foam is yellow or greenish.

A common cause for the appearance of the biliary constituents in the urine is the obstruction of the bile

duct. This may be either from some abnormal growth or merely from inflammation in the passages. The bile is then absorbed by the lymphatics and excreted through the kidneys. The same result may be produced by any abnormal condition of the liver which interferes with the free passage of the bile. A part of the bile may pass from the blood into the tissues, manifesting itself thereby by its characteristic color (icterus).

The biliary coloring matters may be formed in the liver, but they can also be produced by the decomposition of the hemoglobin in the blood and the other tissues of the body, and may pass from here directly into the urine. In this case the urine would contain none of the biliary acids, since they do not appear to be formed outside the liver. A large amount of these acids with the pigments in the urine indicates that the bile comes from the liver (hepatogenous icterus). Some authors have described as a distinct form of icterus that in which the biliary pigments are derived from the blood coloring matters (hematogenous icterus). It seems, however, to be certain that the biliary acids may be absent from the urine even when it contains bile from the liver or gall-bladder.

571. The Production of Artificial Jaundice.—Insert a small cannula into the common bile duct of an anesthetized albino rabbit. Allow a dilute solution of indigo carmine to flow into this from a burette. The conditions are similar to those where the bile is reabsorbed in consequence of some obstruction in the common duct. In a few minutes the mucous membranes show the blue color and it soon is seen under the skin in all parts of the body. Make an autopsy, examining the internal organs to learn how extensive is the diffusion of the color through the tissues.

572. Test biliary urine for the pigments by slowly adding urine from a pipette, to yellow,¹ concentrated

¹ The yellow acid can be made by allowing the colorless acid to stand for some time in a strong light.

nitric acid in a test tube. The acid remains in the bottom, and between the liquids are seen the colored rings, as in 409.

573. To 2 to 3 c.c. of Hammarsten's reagent (410) add a few drops of urine and shake: a green or bluish green color results if bilirubin is present. With minute amounts of bilirubin or when the urine is dark colored, first precipitate the pigments with a little barium chloride, allow the precipitate to settle, pour off the liquid, and stir the precipitate with 1 c.c. of the reagent. The supernatant liquid is green, converted by increasing amounts of the acid mixture, or by yellow nitric acid, through blue and violet to red and yellow.

574. If the urine contains much bilirubin, shake a large test tubeful or more of urine with half an inch of chloroform; pour off the urine and let the chloroform evaporate on a watch glass. The bilirubin is left in small, red prisms. It may be purified by dissolving in chloroform, filtering, and again evaporating. These crystals give the play of colors when moistened with nitric acid. They also dissolve in alkalies, and the solution becomes green on standing (biliverdin).

575. If the urine is dark colored from much urobilin or blood-coloring matters so that the colored rings do not show, test it with *Huppert's* test. Shake a test tubeful of the urine with a small amount of milk of lime, then immediately pass into the liquid a stream of carbon dioxide to remove excess of lime. When it is neutral, filter and wash the precipitate, which contains the biliary pigments. Moisten half of the precipitate on the paper with a drop of moderately strong, yellow nitric acid and observe the play of colors, from red to green. Extract the remainder on a water bath with alcohol slightly acidified with hydrochloric acid; a green color results.

576. In urine which is highly colored with other substances the bilirubin may be identified by Stokvis's test. To 20 or 30 c.c. of urine in a test tube add 5 or 10 c.c. of a 20 per cent. solu-

tion of zinc acetate. Wash the precipitated bilirubin upon a small filter; then dissolve it by the addition of a few drops of ammonia. The liquid which passes through the filter becomes, after standing, brownish green, and shows the spectrum of bilicyanin: an absorption band between *C* and *D* and one between *D* and *E*. If much bile is present the liquid becomes blue upon slightly acidifying.

577. To the urine add a few drops of very dilute tincture of iodine of about the same depth of color as the urine. It turns green. If the iodine is flowed on to the top of the urine by slanting the tube or by dropping from a pipette a green ring is formed.

578. Sucrose-sulphuric acid test for biliary acids (Pettenkofer's).—To 5 c.c. of urine in a test tube add 3 to 5 drops of a 5 per cent. sucrose solution, then let 2 to 3 c.c. of concentrated sulphuric acid flow under the urine. A red ring results. The temperature must be kept at 50° to 70°. The acid produces furfural from the sugar and this reacts with the bile acids.

579. Test the biliary urine for biliary acids by dissolving in it a few crystals of cane sugar, then dipping in it a strip of filter paper. Dry the paper and place on it a drop of concentrated sulphuric acid. In a few seconds it becomes violet, best seen by holding it before a window. Too much sugar gives a brown color.

580. Instead of using concentrated acid make the test with dilute sulphuric acid, as in 391.

It is not advisable to depend upon Pettenkofer's test alone in the urine, as other substances may be present and give reactions similar to those of the bile acids, although their spectra are different. The pure bile acids may, in cases of doubt, be extracted by the following method:—

581. If the urine is highly colored or only a slight amount of bile acids are present, it may be necessary to extract the lat-

ter before testing. Add to the urine lead acetate solution and a few drops of ammonia to make it slightly alkaline. Wash with water the precipitate, which contains the acids, then dry it. Extract it several times with warm alcohol, filtering hot. Make the filtrate alkaline with sodium carbonate, and evaporate to dryness on a water bath. Dissolve the sodium salts of the bile acids from the residue with hot, strong alcohol and filter. The bile salts can be precipitated by adding ether to the cooled alcohol. They become crystalline on standing, or they can be tested for immediately in the alcoholic filtrate with Pettenkofer's or other tests.

HEMOGLOBINURIA AND HEMATURIA.

The hemoglobin is found in the urine in two forms: first, dissolved, no corpuscles being present (hemoglobinuria), and, second, in the corpuscles (hematuria).

The color of urine which contains blood is usually some shade of red, but may be dark brown or even greenish brown when the hemoglobin has been changed to methemoglobin. Very small quantities may not be detected by the eye. The liquid is often more or less cloudy from phosphates, corpuscles, and casts. There may be enough blood present to cause coagulation either in the urinary passages or after the urine is passed.

The free hemoglobin is produced by the destruction of the corpuscles. This may be due to an injection of substances which hemolyze the corpuscles, to the transfusion of blood, to the action of some poisons and in certain infectious diseases, like typhus, also after severe burns. In this case the urine should be tested for hemoglobin. If there is a sediment under these conditions the microscope reveals no corpuscles. The corpuscles also disappear upon standing in ammoniacal urine.

Hematuria, where corpuscles are present, is more common. It is due to hemorrhage in some part of the uri-

nary tract. The corpuscles appear as a sediment and are usually not in rolls. They may be shriveled or swollen from standing in the urine. If the hemorrhage is from the kidney, the blood is usually well mixed with the urine and of a reddish brown color, the reaction being acid. Blood casts may be present, and if they are it is a proof of a renal hemorrhage. This may occur in Bright's disease, also with malignant renal growths or renal calculi.

If the hemorrhage is from the bladder the urine is often alkaline, and clots of blood are common. It may be caused by vesical calculi, by cystitis or villous growths, and by carcinoma.

582. Add a very little blood to highly colored normal urine, and notice that the bands of oxyhemoglobin are visible through the spectroscope, although to the eye there may be no indication of its presence. If the urine is too turbid to examine with the spectroscope, it should be filtered, and if the residue is reddish on the paper this should be washed with 5 c.c. of water and the washings examined.

583. Convert the oxyhemoglobin into hemoglobin as in 351, and notice that the two bands change to one.

584. To urine containing a small amount of blood add enough sodium hydroxide to make alkaline, and heat to boiling. The phosphates of the alkaline earths will be precipitated, and the precipitate will be colored reddish by the hematin from the decomposed hemoglobin. If no blood were present the precipitated phosphates would be white. This test will detect very small amounts of blood in urine. If the liquid is very dark colored, it may be nec-

essary to filter and wash the precipitate before its color can be determined.

585. Mix 2 to 3 c.c. each of 3 per cent. hydrogen peroxide and an acetic acid solution of benzidine, then add as much urine. If blood is present a blue color develops. Pus gives the same result, but not after it has been boiled.

The guaiacum hydrogen peroxide test can also be employed, but its fallacies should not be lost sight of.

586. Examine microscopically the sediment from a urine after recent hemorrhage. Observe the presence of red corpuscles and also the change in their form which takes place after standing.

LIPURIA, OR CHYLURIA.

An abnormal condition of the urine—not uncommon among the inhabitants of the tropics, but more rare among those of cooler climates—is the presence of fat. Lipuria, or the appearance of fat in the urine, may be due to an abscess or fatty degeneration of the kidney; to an excessive amount of fat in the blood, in pregnancy; or to conditions which produce fatty degeneration of other organs, as the liver, and in phosphorus poisoning, whereby the amount of fat in the blood is abnormally increased. The urine is often milky, and, on standing, a creamy layer forms. It contains also the other constituents of the lymph, albuminous substances, etc. In cases of lipuria where only a small amount of fat is present it may appear in the form of drops upon the surface, or it may be present in microscopic globules, either free or in the casts or epithelial cells of the sediment. The globules can be perceived with the microscope and separated by ether.

587. Examine microscopically urine containing fat.

588. To half a test tubeful of urine containing fat add one-fifth its volume of ether away from the vicinity of a flame. Mix by shaking carefully. Allow to stand until the ethereal solution of fat rises to the top. Notice that the urine loses its

milky appearance. Pour off the ether into an evaporating dish and let it evaporate without heating. Dip a strip of white paper in the residue, and notice that a greasy stain remains after drying.

URINARY SEDIMENTS.

Besides the soluble constituents of the urine, there are others which appear as an insoluble deposit upon the bottom of the containing vessel or floating in the liquid. They may be present in the freshly passed urine or may appear after a time. The former are the more important to the physician, although some conclusions as to the condition of the system may be drawn from the latter.

For the collection of these sediments the best method is by the centrifuge, this requiring so little time that the examination can be made before changes have occurred in any of the constituents. If a preservative must be used the urine can be shaken with enough toluene to leave an excess on the surface. The centrifuge is essentially an apparatus where tubes or other vessels can be set in rapid rotation. These tubes swing from their upper end, and as the speed is increased assume a horizontal position. The solid constituents, being heavier than the liquid, are carried by the centrifugal force to the bottom of the tube. The tubes should contain from 15 to 20 c.c., and be rotated three to five minutes at a speed of at least 1500 revolutions per minute.

If a centrifugal apparatus is not at hand, the sediment is best collected by allowing the urine to stand in a conical glass vessel, containing 100 to 200 c.c., until it has settled. Then decant the supernatant liquid or take, by means of a pipette, a sample of the sediment for testing. By closing the upper end of a pipette or pointed tube

with the finger until the lower end is brought in contact with the sediment under the urine, then raising the finger for an instant, the sediment rises into the pipette, from which it can be transferred to a slide or test tube.

Urinary sediments can be divided into two groups: the organized—or anatomical—and the unorganized—or chemical—sediments. Those of the first group are cellular and not single chemical compounds; the latter are of definite chemical composition. Of the unorganized sediments some are soluble in acid and some in alkaline fluids. Their presence depends, therefore, upon the reaction of the urine. They fall naturally into two classes in accordance with their solubility, and may be farther subdivided according to their microscopic appearance. The table which follows gives the most common varieties.

Before examining the sediment, test with litmus paper the reaction of the urine in which it is found. Then place a drop of urine containing the sediment on a glass slide, cover with a cover glass, and examine microscopically with a 16-millimeter objective. The microscopic examination should be made before the liquid evaporates and leaves the soluble compounds on the slide. A higher power may be used afterward if necessary, but generally the low power is preferable. Chemical reagents may be applied on the slide after removing the excess of urine by a piece of porous paper. Place one drop of the reagent on the slide by the side of the cover glass. It will flow under the cover glass, and its action can be observed with the microscope as it comes in contact with the different sediments. Care should be taken not to allow the reagents to touch the microscope stage. If a low power is used without a cover glass these tests may be made in

URINARY SEDIMENTS.	
ORGANIZED, OR ANATOMICAL.	UNORGANIZED, OR CHEMICAL.
Pus.	
Mucus.	
Epithelium.	
Blood-corpuscles.	
Casts.	
Bacteria.	
Spermatozoa.	
	IN ACID URINE.
	<i>Crystalline.</i>
	<i>Non-crystalline.</i>
	Uric acid.
	Acid urates.
	Calcium oxalate.
	(envelope shaped).
	(dumb-bell).
	Triple phosphate
	(in very weakly
	acid urine).
	Fat.
	<i>Crystalline.</i>
	<i>Non-crystalline.</i>
	Triple phosphate.
	Calcium oxalate.
	Ammonium
	urates.
	Calcium carbonate.
	Calcium oxalate.
	<i>Crystalline.</i>
	<i>Non-crystalline.</i>
	Calcium sulphate.
	Tyrosine.

a flat watch crystal. Where large quantities of a reagent are employed, as in testing pus with an alkali, the ordinary chemical vessels are to be used.

Urine containing pus is turbid when freshly passed, and gives the albumin reactions. When much pus is present it may fall to the bottom as a thick sediment. Small quantities may remain suspended for a long time. In urine of an acid reaction the pus-corpuscles can be seen. They are circular and colorless, about twice the diameter of the red blood-corpuscles. They appear granular, but when brought in contact with acetic acid the granulation disappears and the nuclei, of which there are two or three, become visible. (Plate III, 13.) When the urine becomes alkaline, either by fermentation or by the addition of a fixed alkali, the corpuscles disappear and the mass becomes very sticky and gelatinous, so that it can be drawn out by a glass rod into long threads. The turbidity of urine which contains pus resembles that from urates or from the earthy phosphates. It does not disappear, however, like the former, by warming, nor, like the latter, upon the addition of acids.

The source of the pus in the urine may be anywhere in the urinary tract. When it is from the kidney the urine is apt to be acid in reaction, and round-celled epithelium or casts may be present. When it is from the bladder the urine is usually alkaline. It may be due to simple inflammation or to some deep-seated affection of the tissues.

589. Examine microscopically urine containing pus. Remove the excess of liquid around the cover glass by means of a piece of filter paper. Put a drop of acetic acid on the slide and let it run under the cover glass.

Notice the change in the appearance of the corpuscles.
Sketch.

590. Show that the turbidity does not disappear upon warming or upon acidifying.

591. If enough pus can be obtained, make Donné's test by allowing it to settle, then, after decanting off the urine, making it alkaline with sodium hydroxide. The mass becomes extremely viscid, as is shown by stirring or pouring.

592. Show that the pus responds to the albumin reactions.

Mucus as a sediment is in the form of a slimy, viscid liquid, sometimes showing the mucous corpuscles. Its significance has been explained before. It can be made more visible by adding a little tincture of iodine, which colors it brownish. The addition of acetic acid to the urine precipitates mucin as a fibrous mass.

The epithelial cells, being continually thrown off from mucous surfaces, are normally present in small numbers in the urine. In such cases they are usually from the bladder and urethra, and, in women, from the vagina. A large increase, however, is indicative of a diseased condition of some part of the urinary system. The cells from different parts of the system are not all of the same shape. (Plate III, 14.) Several different forms are to be sought: the squamous, or pavement epithelium; the round celled; and the long, or spindle celled, epithelium. The squamous epithelium is composed of large, flat, somewhat irregular cells with a distinct nucleus. They may be found singly or united, like the stones of a pavement. They occur chiefly in the outer layers of the

mucous membrane of the vagina and bladder. The round celled epithelium has smaller cells with a nucleus and nucleolus and are found especially in the tubules of the kidneys. They are also found in the deeper layers of the mucous membrane of other tissues, such as the bladder, urethra, and pelvis of the kidney. They are somewhat larger than the pus corpuscles, and the nucleus can be seen without clearing by acetic acid. The long celled epithelium is narrow and somewhat irregular, with a nucleus visible without staining. They are found in the outer layer of the membrane of the renal pelvis or in the deep layers of the bladder, ureters, and urethra.

Although the presence of a single kind of epithelial cells in the urine may give an indication of their origin, still their occurrence in different tissues often renders this a matter of doubt. The condition of the cells, however, may furnish information of the pathological changes which have taken place. If they appear disintegrated or contain fat globules, their origin is from the locality of some degenerative process, often of a chronic nature.

Blood corpuscles are not normal in urine. In freshly voided urine they may retain their normal shape,—that of a biconcave disk. (Plate III, 13, *d.*) In acid urine, especially where the specific gravity is high, they shrivel after a time, the margins become irregular. In dilute urine and where the reaction is alkaline the corpuscles swell, and become biconvex or spherical. If there is much blood the liquid is reddish, but a slight amount may escape detection by the unaided eye. When it is present the albumin reactions can always be obtained.

By urinary cast is meant an irregularly cylindrical mass, composed of various materials, which have been formed in the tubules of the kidney, and hence are of

about the same size as the tubules. Opinions vary as to the cause of their formation, but most casts appear to be due to the coagulation of the serum which passes into the renal vessels owing to some pathological condition. The presence of anatomical elements—such as epithelium, pus, blood, and fat—or their decomposition products in the coagulated mass gives the different varieties of casts.

Epithelial casts are not very common. They consist of cylindrical shaped masses of round epithelial cells which are thrown off from the tubules by some inflammatory process. The cells may appear normal or they may be more or less decomposed and of a granular appearance, or they may contain minute fat globules. The cells sometimes seem to compose the whole cast and sometimes to be scattered over its surface. (Plate III, 16.) When present, they indicate inflammation of the kidney. When the cells are degenerated, the indications are that the condition is chronic or has existed for some time.

Blood casts consist of coagulated blood often containing so many red corpuscles that they are dark and non-transparent. They may be formed whenever hemorrhage occurs in the urinary tubules, and are the best evidence of this. They are quite rare, and may be obscured under the microscope by the free blood corpuscles.

Casts of pus are also very rare, but pus corpuscles are not infrequently seen in other varieties of casts.

By the decomposition and metamorphosis of epithelium, blood or pus cells in casts, the so-called granular casts have their origin. They vary greatly in size, shape, color, and in fineness of granulation. (Plate III, 15.) The finely granular cannot be easily seen except with a high power of the microscope, although the coarsely

granular may be observed with a low degree of magnification. They often contain unaltered epithelium, leucocytes, and fat globules. Granular casts indicate degeneration or a long continued pathological condition of the kidney.

Occasionally casts of fat globules are observed. They result from farther metamorphosis of the granular casts. (Plate III, 18, a.)

In diseases of the kidney, like interstitial suppurative nephritis, where bacteria are abundant, casts composed of these organisms are often seen. They resemble granular casts, but are not destroyed by mineral acids and caustic alkalies, as are the granular casts. High powers of the microscope should be used in their examination.

Hyaline casts are almost transparent or at most show only a very fine granulation. On account of their great transparency they are extremely difficult to perceive. They may be colored yellow by adding a solution of iodine. As a rule, these casts can be stained so as to be more readily recognized by mixing a few drops of a solution of one of the water soluble coal tar colors with the urine on the slide. In shape they are usually long and narrow. Besides these narrow hyaline casts, which probably are formed in the smaller tubules, there is sometimes found a broader variety. (Plate III, 17.) These have an indented edge and, in consequence of being more highly refractive, can be seen more easily than the narrow ones. They are called waxy casts. They often give the amyloid reaction,—a brown color with iodine, turning blue to violet upon acidifying with sulphuric acid. They are doubtless formed in the renal pyramids. The narrow casts dissolve readily in acetic acid, but the waxy casts remain in it for some time.

Hyaline casts not infrequently have anatomical elements—blood and pus corpuscles, epithelium, etc.—clinging to the surface or included within the mass.

The origin of the hyaline casts seems to be due to the coagulable elements of the blood. It is doubtful if they are ever present in urine which has not been albuminous. Their presence, consequently, is indicative of the existence of albuminuria. They may be the best evidence of such a condition as interstitial nephritis, where the amount of albumin is small.

Whatever variety of cast may be present in urine, it shows, without any doubt, that there is a pathological condition of the kidney and that the accompanying albumin is of renal origin.

Besides these cylindrical casts there sometimes appear in the urine the so-called cylindroids. These are flat or ribbon shaped, rather than cylindrical. They are usually about the diameter of casts, but longer, and resemble in their transparency and solubility the hyaline casts, their composition being probably the same. They are found in nephritis and congestion of the kidneys, also in cystitis. They do not seem to be characteristic of any pathological condition of the kidneys, but rather of some irritation of the lower urinary tract which has extended to the kidneys.

All casts are decomposed by bacterial action. The examination should, therefore, be made as soon as possible after the urine is passed and the casts have settled. This time may be shortened to five minutes by the use of the centrifuge. Without this it will be necessary to let the urine stand several hours or over night.

593. To examine urine for casts a few drops from the sediment obtained from standing in a conical glass or

from the centrifuge is placed upon the microscope slide; one with a shallow cell on top is best. Cover it with a cover glass and remove the liquid outside by filter paper. Focus on the sediment, using a 4 mm. objective, then cut off nearly all light from below. When transparent or hyaline casts are sought for swing the mirror to one side and upward and throw the illumination upon the slide obliquely or use a small diaphragm. They will be more plainly visible by this means than with a strong illumination. After the casts have been detected their cylindrical shape can be shown by inclining the stage of the microscope so that they roll in the liquid. Sketch them.

BACTERIA.

The freshly voided normal urine contains no bacteria. They may be present, however, under abnormal conditions, and will soon appear in normal urine upon its standing exposed to the air. On account of the large amounts of organic matter dissolved in the urine, it furnishes a medium in which micro-organisms readily grow. This occurs even in the bladder if they are introduced from the outside, as, for example, by means of an unclean catheter. Urine containing bacteria is cloudy and is not cleared by filtration.

The non-pathogenic organisms are found in putrefying or decomposing urine. This is usually not acid and often is strongly ammoniacal. They may be found thus in the urine of cystitis where ammoniacal fermentation is excessive. Some of these are of large size and can be observed with a 4 mm. objective without staining. (Plate II, 8.) The pathogenic organisms are such as the pus organisms, the diplococcus of gonorrhea, and also

the bacillus of tuberculosis and the organisms of infectious diseases. They can be examined and isolated by the common bacteriological methods.

SPERMATOZOA.

These may be found in the urine of males after coitus or pollution. They may be present in some diseases, like typhoid, and are constantly found in spermatorrhea. By straining during defecation there may be a slight emission of semen, and consequently the spermatozoa be mixed with the urine. They are readily recognized by their characteristic shape under the microscope,—a flattened oval head united with a long thread like body and tail. (Plate III, 18, c.) They are most abundant in the first and last portions of the urine.

In freshly voided urine they may have some motion, but this soon ceases. Acids and alkalies, as well as pure water, stop it immediately. Spermatozoa resist putrefaction and the action of chemical reagents, even that of strong acids or alkalies.

URIC ACID AND URATES.

The properties of these compounds have been given before. As a sediment, the free acid and its salts differ from all others in being colored yellow to brown. They are not abnormal in urine unless they are present as solids when the urine is passed, or are deposited within a few hours, since normal urine throws down uric acid after long standing. The precipitation of these compounds is largely effected by concentration or an increase in the acidity of the urine. The normal or dibasic urates are readily soluble in water, and do not occur in

sediments. When the acidity of the liquid is increased, either by fermentation or by the addition of an acid, half the base is taken from these salts, leaving the mono-basic or acid urates, which are soluble with much more difficulty. If the acidity becomes still greater, all the base is removed, leaving the free acid, which is only very slightly soluble in water. Of course, a decrease in the volume of water would be accompanied by a corresponding increase in precipitated uric acid and its compounds. Hence a sediment of these may appear in the urine without signifying that an increased quantity has been formed in the body. Thus, they are common in fevers, when the urine is of small volume and concentrated. Less uric acid is formed in the body with a vegetable diet than with one of meat.

Uric acid and urates as sediments occur mostly in acid urine and can be usually identified microscopically. (Plate II, 11.) The color is characteristic. The acid is always crystallized, commonly oval or diamond shaped, sometimes visible to the naked eye, often in clusters or rosettes. The urates are commonly salts of sodium, potassium, or ammonium. They may be amorphous when examined with high powers. The so-called "brick-dust" sediment is a mixture of the sodium and potassium urates. Sodium urate is also found in fan shaped clusters or irregular groups of fine crystals, and sometimes in granules. (Plate II, 8.) Ammonium urate makes up the "thorn apple" crystals: brown, spherical masses covered with curved spicules. (Plate II, 9.) The urates can be differentiated from other sediments by being soluble on gently warming the liquid, as well as in alkalies. The urates, as well as the free acid, give the murexide reaction (499). Uric acid is especially impor-

tant when found as a sediment, from its tendency to form calculi. The same is true, to a less extent, of the urates.

CALCIUM OXALATE.

This salt is most frequent in acid urine. It may exist in two forms: the crystalline, or "envelope shaped," and the "dumb bell shaped." Its appearance under the microscope affords the best methods of identification. (Plate II, 10.) The crystalline form consists of octahedral crystals. They are never large, often being smaller than a red blood corpuscle. When sufficiently magnified, they have somewhat the appearance of the back of a square envelope, the crossed lines being formed by the angles of the crystal. In the shape of the crystals they resemble some forms of triple phosphate, from which they can be distinguished by their insolubility in acetic acid and by their smaller size. The amorphous form of calcium oxalate is disk shaped, with a contraction on opposite sides, so that it somewhat resembles a dumb bell. Calcium carbonate has much the same form, but dissolves in acids with effervescence. Calcium oxalate is insoluble in acetic, but soluble in hydrochloric acid. The dumb bell form gives rise to calculi of the bladder.

Oxalic acid and its salts are found in many fruits and vegetables,—like tomatoes, celery, rhubarb, etc.,—and when these are eaten it appears as the calcium salt in the urine. It is also produced in the body from certain foods,—as from large quantities of nitrogenous foods or from the carbohydrates, where the oxidation is not complete. A small amount, then, may be normal, and if it is transitory is of no great consequence. If the excretion is continual it is due probably to some constitutional weakness.

PHOSPHATES.

The phosphates of the alkalies, being readily soluble in water, do not appear as urinary sediments. The phosphates of calcium and magnesium are insoluble in water or alkalies, although they dissolve in acids. They, consequently, appear as sediments whenever the urine becomes alkaline, but are not found in acid urine unless the acid reaction is very faint. They can be distinguished from other urinary sediments by dissolving in acetic acid without effervescence.

Triple phosphate, NH_4MgPO_4 , is a salt of phosphoric acid having two bases—ammonium and magnesium. When it is made by precipitating a phosphate by ammonia and magnesium sulphate the crystals are usually stellate or snow-flake formed. As it is slowly formed in the urine, however, they are more commonly in the form of rhombic prisms. The terminations of the prisms are commonly truncated; so that the crystals have a shape which approaches that of the end of a coffin, and this gives rise to the common appellation: "coffin lid crystals." (Plate II, 8.) The angles may not be so truncated and the long axis of the crystal may be so much shortened that it assumes the form of an octahedron, like the calcium oxalate. Unlike the latter, it is soluble in acetic acid. Calcium phosphate in the urine is usually amorphous, and always colorless. It is formed when the urine becomes alkaline in the absence of ammonia. To the unaided eye it resembles pus, but differs from it in its solubility in acids. In acid urine the acid phosphate, CaHPO_4 , may crystallize in long prisms, usually in clusters. Tribasic calcium phosphate, $\text{Ca}_3(\text{PO}_4)_2$, is colorless and amorphous. (Plate II, 7.)

The presence of phosphates may be due to an excessive formation in the body, and they are then usually accompanied by systemic disturbances. Alkalinity of the urine causes their appearance when there is no excess. This may be from the food or medicine, from an increase in the alkalinity of the blood, or from fermentation. Excessive mental work is often accompanied by phosphatic sediments. Their long continued presence may excite fear of the formation of calculi. Their temporary appearance is a matter of no grave significance. In urine which has stood for a time after its passage they are the most common of the sediments.

594. Drop dilute ammonia into clear normal urine until it is very slightly turbid, and after it has settled examine the sediment with the microscope. It is a mixture of the amorphous calcium phosphate and crystalline triple phosphate. To obtain a larger amount of the latter add to the urine a little magnesium sulphate before it is made alkaline.

Note difference in crystalline form of the precipitate formed slowly in this manner and when the precipitation is rapid from quickly making alkaline with ammonia. They are both the same compound. Sketch the principal modifications.

595. Precipitate sodium phosphate with magnesium sulphate after making alkaline by ammonia. Notice the difference in the shape of these stellate crystals under the microscope and those usually formed in the urine. Try the solubility of both forms in acetic acid.

596. Make normal urine alkaline with sodium hydroxide and examine the precipitated calcium and mag-

nesium phosphates with the microscope. Try their solubility. Give formulas.

597. Let 100 c.c. of urine stand for several days until ammoniacal fermentation has developed. Examine the sediment with the microscope. How many compounds are seen and what are they? Is all the ammonia combined in the triple phosphate? Proof? Is the alkalinity due entirely to ammonia or partly to a fixed alkali? Try it (470).

CALCIUM SULPHATE.

This does not often occur as a sediment. It may be found in acid urines as long prisms united in clusters. (Plate II, 12, *a.*)

598. Prepare crystals of calcium sulphate by precipitating a rather dilute solution of calcium chloride with a few drops of sulphuric acid. Dissolve the precipitate in boiling water, filtering hot if all does not dissolve. It will reprecipitate upon cooling. Examine with the microscope and sketch the crystalline forms.

CALCIUM CARBONATE.

This compound is often found in alkaline urine with calcium phosphate. It appears as a sandy powder which, when examined microscopically, is seen to consist of spherical bodies formed of concentric layers or to have the dumb bell shape like calcium oxalate. (Plate II, 9.) It dissolves readily in acetic or other acids, with the evolution of carbon dioxide gas.

599. Allow a sample of urine to stand several weeks and ferment at room temperature. The sediment usu-

ally contains, besides the phosphates and bacteria, ammonium urate and calcium carbonate. Explain their formation. Sketch the chief forms.

TYROSINE.

Tyrosine is not often found as a sediment because of its solubility in water, but it sometimes appears as such, though never in a normal condition of the system. It crystallizes in minute needle shaped crystals, which are usually aggregated into clusters or sheaves. (Plate II, 12, c.) Its microscopic appearance is the best means of identifying it. The chemical tests have been given.

Tyrosine in the urine has the same source as in digestion—the decomposition of protein compounds. It is improbable that it comes from the intestine, but rather from other parts of the system. It is indicative of retrograde metamorphosis of the nitrogenous tissues. Thus, it is present in acute atrophy of the liver, in suppurative processes, and in phosphorus poisoning, which is accompanied by degeneration of the liver. Leucine is sometimes found at the same time. (Plate II, 12, b.)

FAT.

The appearance and significance of fat in the urine (lipuria) has already been discussed.

SYSTEMATIC TESTING OF URINE.

In the systematic testing of urine the course is often varied, as the symptoms may point to the likelihood of the presence or absence of certain substances. The quantitative tests may be made use of or not according to

circumstances. The following are the determinations which are most important, with the tests which may be employed :—

Amount passed in twenty-four hours.

Color
Transparency } Normal or abnormal.
Odor } If the latter, what is the cause?

Specific gravity at 15.5° (60° F.) (469).

Chemical reaction to litmus.

If alkaline, is it from ammonia or fixed alkalies?
(470).

Titratable acidity (471).

Hydrogen ion concentration (476).

Alkali tolerance (474).

Acidosis (478).

Nitrogen, total (490, 492, 493).

Nitrogen partition.

Non-protein nitrogen.

Ammonia (477, 495).

Urea (485, 486, 487, 488).

Uric acid (503, 504).

Creatinine (512, 513).

Creatine (514).

Amino acids (495).

Protein nitrogen.

Albumin.

Qualitative reactions (533-536).

Quantitative determination (537, 538,
539, 540).

Proteose and peptone (543).

Glucose.

Qualitative reactions (548).

Quantitative determination (549, 550, 551, 552, 553).

Lactose (568, 569).

Acetone bodies.

Acetone, qualitative tests (555-562).

quantitative determination (563, 567).

Acetoacetic acid, qualitative tests (564-566).

quantitative determination (567).

Beta-oxybutyric acid (567).

Bile.

Biliary acids (578-581).

Biliary pigments (572-577).

Blood (582-586).

Sulphates.

Inorganic, qualitative test (526).

Organic including indican, qualitative tests (526, 529, 530).

Quantitative determination (532).

Phosphates.

Quantitative determination (521).

Chlorides (516).

Identification of sediments, if present.

I. UNORGANIZED.

(A) *Crystalline.*

Uric acid.

Calcium oxalate.

Calcium phosphate.

Triple phosphate.

Other rarer compounds.

(B) *Amorphous.*

Urates.

Phosphates, etc.

II. ORGANIZED.

Pus.

Mucus.

Blood corpuscles.

Bacteria.

Spermatozoa.

Epithelium: kind and probable source.

Casts: kind and probable cause.

The proof of the presence of any abnormal constituent should not be allowed to rest upon one test, but several should be tried.

600. **The Effect of Food on the Composition of the Urine.**—Let a number of subjects each select food of a different class and eat only this for twenty-four hours, collecting all the urine for the period. The following dietaries will give a variety:

1. Largely animal.
2. Vegetable.
3. Rich in purins, sweetbreads, etc.
4. Purin free—milk, eggs, wheat bread, butter, cheese.
5. Low in nitrogen.
6. No food.

Determine volume, specific gravity, color, reaction; amounts of nitrogen, uric acid, phosphoric acid, urea, creatinine.

URINARY CALCULI.

The constituents of calculi are the same as those of the chemical sediments, and the causes which give rise to the formation of the latter will also favor the production of calculi in the bladder. To these various names are applied, according to their size: sand, gravel, stone, and

calculi, or concretions. They vary from the microscopic to aggregations as large as an orange. They are generally not composed of a single material, but have at the center a nucleus, and this is surrounded by layers, often of two or more compounds in alternation. The nucleus may be a mass of foreign matter, or it may be a clot of blood or a particle of one of the sediments around which material, perhaps of a different kind, has been deposited.

Uric acid concretions are the most common. They are brown in color, rough of surface, and brittle. The form of the crystals cannot be seen, but they give the murexide reaction. They dissolve in sodium, or potassium hydroxide, from which solutions the uric acid may be precipitated in the crystalline form by the addition of a mineral acid. Uric acid calculi are formed only in an acid urine.

The urates are often found mixed with the uric acid deposits or with those of calcium oxalate. The ammonium salt is the most abundant. They are generally small, grayish, and rather soft. They give the murexide test. They are deposited from acid urine, except the ammonium urate, which is formed in an alkaline solution.

Calcium oxalate concretions are commonly of large size and are very hard. The surface is rough and warty. They are called "mulberry calculi" from the resemblance of the surface to that of the fruit. The urine is generally acid, unless where the presence of the stone has produced cystitis. They are often dark in color from the blood which has been incorporated with them.

The phosphates can only be present in calculi when the urine is alkaline. They are generally rather soft and easily broken. Calcium phosphate has a chalky appearance. Triple phosphate, NH_4MgPO_4 , is found with

other substances. It is more commonly on the outside of the stone, being precipitated by the alkaline reaction in the bladder. A mixture of the triple phosphate and calcium phosphate is fusible with the blow pipe and is known as the "fusible calculus."

Calcium carbonate calculi are not common, although found occasionally. It is frequently met with in the phosphatic deposits.

The analysis of calculi is made by the use of chemical methods. The stone should be broken or, better, if it is large enough sawed through the middle. This shows the layers of which it is composed and the nucleus. If there appears to be any difference in the layers, they should be tested separately. Heat a piece upon platinum foil or thin porcelain and notice whether it fuses and whether it is combustible or not. If it fuses it is an indication of a phosphate of calcium and triple phosphate. If it is combustible it consists of organic compounds.

Blackening when ignited is evidence of organic matter, but if slight it may be merely mucus arising from irritation of the bladder, and not an essential part of the calculus. Ignition on the foil will divide the constituents into two classes, although both may be present.

COMBUSTIBLE, OR
ORGANIC.

1. Uric acid.
2. Ammonium urate.

INCOMBUSTIBLE, OR
INORGANIC.

1. Calcium phosphate.
2. Calcium oxalate.
3. Calcium carbonate.
4. Triple phosphate.
5. Urates of K, Na, and Ca.

If it is composed largely or entirely of organic matter try the murexide test (499) for uric acid and urates. If

inorganic compounds are present, powder a piece and treat in a test tube with 2 or 3 c.c. of dilute hydrochloric acid. Carbonates dissolve with effervescence of carbon dioxide gas, the others without. Warm, if necessary. Filter, if it does not give a clear solution. To one-fourth of the filtrate in a test tube add sodium hydroxide until it is alkaline, and test for ammonia by hanging in the tube a strip of moist red litmus paper, being careful that it does not touch the side of the tube which is wet with the sodium hydroxide. The tube can be allowed to stand corked over night or the ammonia gas can be expelled from the liquid by boiling. If present it will turn the paper blue.

To the remainder of the solution in hydrochloric acid add ammonia until it is alkaline, acidify with acetic acid, and boil. If there is a precipitate, filter.

Precipitate is calcium oxalate. Test after washing and drying by heating to a bright red heat on platinum foil. After cooling it should turn moist red litmus paper blue.

To the filtrate add ammonium oxalate, boil, and, if there is a precipitate, filter while hot.

A white precipitate shows calcium, probably originally present as phosphate or carbonate.

The filtrate is to be tested for magnesium and phosphoric acid. For Mg make one-half alkaline with ammonia and if the liquid remains clear, add sodium phosphate. A fine, white crystalline precipitate with either reagent indicates Mg. For phosphoric acid make remainder acid with strong HNO_3 and add ammonium molybdate. A yellow precipitate appears.

Urates of K, Na, and Ca can be found by boiling the powdered calculus in water, filtering and testing the filtrate by the murexide test. Or if it is evaporated to dryness and the residue is ignited on platinum the sodium and potassium will remain as carbonates, giving an alkaline reaction to litmus paper.

THE METRIC SYSTEM.

In all work in modern chemistry the metric system of weights and measures is employed. The unit of length is the meter (39.37 inches); of weight is the gram, which is the weight of 1 c.c. (or mil) of water at 4°; and, of capacity, the liter, which has the volume of 1 cubic decimeter.

Measures of Length.

10 millimeters	= 1 centimeter.
10 centimeters	= 1 decimeter.
10 decimeters	= 1 meter.
10 meters	= 1 decameter.
10 decameters	= 1 hectometer.
10 hectometers	= 1 kilometer.

Measures of Weight.

10 milligrams	= 1 centigram.
10 centigrams	= 1 decigram.
10 decigrams	= 1 gram.
10 grams	= 1 decagram.
10 decagrams	= 1 hectogram.
10 hectograms	= 1 kilogram.

Measures of Volume.

10 milliliters	= 1 centiliter.
10 centiliters	= 1 deciliter.
10 deciliters	= 1 liter.
10 liters	= 1 decaliter.
10 decaliters	= 1 hectoliter.
10 hectoliters	= 1 kiloliter.

The following are especially to be remembered:—

One gram is the weight of 1 cubic centimeter of water measured at 4° C.

A liter contains 1000 cubic centimeters and a liter of water weighs, therefore, 1000 grams.

The following are convenient in the conversion of the weights and measures of one system into another:—

1 meter	= 39.37 inches.
1 foot	= 0.304 meter.
1 liter	= 61.03 cubic inches = 1.06 U. S. qts.
1 liter	= 33.81 U. S. fluidounces.
1 gram	= 15.43 grains.
1 grain	= 0.0648 gram.
1 ounce (apoth.)	= 31.1 grams.
1 ounce (avoirdupois)	= 28.35 grams.
1 pound (apoth.)	= 373.2 grams.
1 pound (avoirdupois)	= 453.6 grams.

REAGENTS.

The preparation of most of the special reagents has been given in the text. The following include the most common of the others used. The percentages are by weight and approximate only.

Acids:

Sulphuric acid, 10 per cent.; pour 1 volume of the concentrated acid into 18 volumes of water.

Hydrochloric acid, 5 per cent.; 1 volume of concentrated acid and 6 of water.

Nitric acid, 10 per cent.; 1 volume of concentrated acid and 6 of water.

Acetic acid, 6 per cent.; dilute the concentrated with water.

Tannic acid, 10 per cent.; dissolve 10 grams in 100 c.c. of water.

Picric acid, a saturated aqueous solution.

Other inorganic reagents:

Ammonium hydroxide, 5 per cent.; dilute 1 volume of the concentrated solution with 4 of water.

Make the following by dissolving about 50 grams of the solid in 1 liter of water:—

Sodium hydroxide.

Ammonium oxalate.

Potassium ferrocyanide.

Potassium ferricyanide.

Lead acetate.

Sodium phosphate.

Ferric chloride.

Cupric sulphate.

Mercuric chloride.

Silver nitrate (a 1 per cent. solution answers in many tests).

Iodine, a 1 per cent. solution of potassium iodide with enough crystals of iodine to give the color desired.

Indicators:

Congo red; 0.5 gram in 100 c.c. of 10 per cent. alcohol.

Methyl violet; 0.5 gram in 100 c.c. of water.

Tropaeolin OO; 0.5 gram in 100 c.c. of water.

Phenol phthalein; 1 gram in 100 c.c. of 95 per cent. alcohol.

Sodium alizarine sulphonate; 1 gram in 100 c.c. of water.

Dimethyl-amido-azobenzene; 0.5 gram in 100 c.c. of 95 per cent. alcohol.

Methyl red; a saturated solution in 50 per cent. alcohol.

Neutral red; 1 gram in 100 c.c. of 50 per cent. alcohol.

Paranitrophenol; 1 gram in 100 c.c. of 95 per cent. alcohol.

APPENDIX.

THE OBTAINING AND PRESERVATION OF TEST MATERIALS.

Although obtaining materials for a laboratory course in biological chemistry is easy in a medical school where the hospital can be drawn upon it may not appear so simple to teachers who do not have these facilities. As far as possible the feasibility of getting materials has been borne in mind in planning this work. The stability of animal substances also raises a doubt in the minds of some teachers as to possible difficulties arising from this fact.

Some articles can be bought in a stable form. Such are dried serum albumin and egg albumin, peptone (which usually contains proteoses), ground hempseed, ground malt, etc. Except for practice there is no reason for preparing them. Malt can be made by keeping moistened barley in a dark, warm place until the rootlet is about the length of the grain, then drying.

Of the enzymes pepsin can be bought of a high grade. The pancreatic enzymes are for sale but their activity is often uncertain; it is better to use the fresh glands. Very active urease can be bought from the Arlington Chemical Co., who also can furnish the ground meal from the soy bean or jack bean. Glycogen can be bought but is expensive; it can better be prepared in the laboratory, especially as its preparation brings out so many valuable facts.

Much material must come from the slaughter-house. With a little instruction one of the workmen will lay aside stomachs, gall-bladders or such tissues as the pancreatic and submaxillary glands. If the freshly drawn blood is beaten with a bundle of sticks the fibrin coagulates and, in the laboratory, can be first separated from the blood, then washed white by letting water run into the bottom of the vessel through the night.

Preservatives can be used against bacterial decomposition. Shaking an animal protein solution with a little chloroform so that a few drops remain undissolved will prevent putrefaction for months. Mixing with a little toluene so that a film lies on the surface prevents bacterial decomposition for a long time and does not injure the organic compounds present. Formaldehyde preserves well but it has a reducing action and hardens proteins so it is not as advisable. Solids like fibrin can be preserved in glycerol or it can be dried in a current of air and will keep indefinitely; soaking in water makes it ready for digestion experiments. Metallic antiseptics, like mercuric chloride, should, as a rule, be avoided.

Physicians are usually generous in co-operating by furnishing to serious students pathological materials which they may meet in their practice, like urine and gastric juice. In default of such sources they can be imitated by adding pathological constituents to normal urine, or by mixing with water the compounds which might be possible in gastric juice. In such substitution it is very important that the amounts used and their combinations shall not differ from those which do actually occur.

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